Signaling After the First Bite: The Role of Insect Elicitor and Jasmonic Acid Metabolism In Shaping Plant-Insect Interactions

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Chapter 1

General Introduction

Arjen van Doorn

General Introduction

Evolutionary history of herbivory

Some 400 million (m) years ago, soon after the first land plants evolved, arthropods started feeding on the basic structures of these plants; fossil evidence shows arthropod damage on sporangia and stems dated between the latest Silurian and early Devonian; corresponding to 417 to 403m years ago (Labandeira, 2007). Since then, plants have evolved new tissues, and insects have adapted to feed on them. Since this first, 400m- year-old bite, plants and insects have evolved alongside each other, and today there are ca. 4 to 6m insect species, of which about 45% are phytophagous and feeding on the approximately 300,000 plant species there are on this planet (Novotny *et al.*, 2002). To put this time frame into some perspective, we, as the last surviving species in the *Homo* genus diverted from Chimpanzees ca. 5m years ago (Kumar *et al.*, 2005), ca 65m years after primates evolved (Chatterjee *et al.*, 2009).

Many insects have specialized themselves to feed on a limited number of plant families; it is estimated that 90+% of all herbivores are specialized, and restricted to feeding on less then three different plant families (Futuyma and Gould, 1979). The first to systematically perform experiments with plants and herbivores was Ernst Stahl, who described in 1888 that compounds from plants are responsible for resistance against herbivorous snails, and that by extracting leaf tissue with different solvents, a non-host plant can be changed to a host, and vise versa. About 70 years later, Gottfried Fraenkel developed Stahls ideas further, proposing that plant secondary metabolites are important in their defense against various biotic attackers (Fraenkel, 1959). In their pivotal 'plants and butterflies' paper, Ehrlich and Raven (1964) laid the fundaments for the theory of chemical co-evolution, proposing that the interaction between plants and herbivores have shaped chemical diversity in plants, and have driven speciation events. Indeed, 50 years after these groundbreaking studies the field of chemical ecology have evolved and shown in great detail that secondary metabolites plays indeed a very important role in plant defense, and compounds classes such as terpenes, alkaloids and quinones have been demonstrated to play an important role in the defense against herbivores, and adaptation to these defenses is thought be responsible for herbivore specialization.

Induced defenses

Being loaded full of toxic and deterrent compounds might provide great protection against insects, but it is a double-edged sword; plants also benefit from insects such as pollinators and predatory insects that feed on herbivores. Moreover, the production of these plant secondary metabolites is costly in terms of energy and resources. Consequently, mechanisms that balance

defense against foes while not poisoning friends are required. Such a mechanism was first reported in the interaction between plants and pathogens, where Smith and his co-workers observed in 1911 that *Bacterium tumefacians* (now called *Agrobacterium tumefacians*) bacteria were unable to produce tumors on plants which had been previously exposed to the same bacteria, showing that the plant can induce resistance to an attacker. Many studies have since observed similar responses between different plants and phytopathogens. The idea of plant immunity however, was initially met with great criticism [e.g.(Silberschmidt, 1931)], and it was not until 1933, when Kenneth Chester reviewed the extensive literature regarding this subject, proposing that acquired 'plant immunity' was a widespread phenomenon, even though the mechanisms were unknown at that time. Since then, induced defenses have also been described in algae diatoms: when algae are attacked by a predator, they change their shape in a way which hinders indigestion by the predator (Gilbert, 1966).

In the context of herbivory, induced defenses allow plants to elicit a targeted response to a feeding insect, and one of the first to describe a wound-induced signal was Clarance 'Bud' Ryan, who described proteinase inhibitors (PIs) as a wound-induced trait (Green and Ryan, 1972). Because the response to herbivory consists of multi-faceted mechanisms, central organization is essential, and is mediated in plants by phytohormones. The phytohormone which plays a predominant role in the defense against insects is jasmonic acid (JA) and its derivatives, jasmonates, while salicylic acid (SA) and ethylene moderate the jasmonate-mediated response via cross-talk mechanisms (Koornneef and Pieterse, 2008; Rojo *et al.*, 2003). In response to wounding, the tissue around the wound will accumulate jasmonates within a minute (Glauser *et al.*, 2008), and these jasmonates will then activate a signal transduction pathway leading to the activation of the defense response. This response consists of both direct and indirect defenses: in addition to metabolites already present, the plant may accumulate toxic, deterrent, antifeedent, and predator- or parasitoid-attractive metabolites that should fend off herbivores once they feed on the induced material. To reduce access to tissues, or to deliver defense metabolites, some plant species also induce the formation of thorns or trichomes. Indirect defenses attract natural enemies of herbivores and include volatile organic compounds (VOCs), extrafloral nectar or food bodies, and shelter (Alborn *et al.*, 1997; Heil *et al.*, 2001; Kessler and Baldwin, 2001). Because the way to a predator's heart is through its stomach, it is essential that non-nutritious indirect defenses (e.g. VOCs) are an honest cue, and are only released when herbivorous insects are present on the plant. The wounding events a plant experiences in nature are not exclusively caused by feeding herbivores: Plants can also be mechanically wounded by a myriad of other factors, and must distinguish mechanical from herbivore damage.

Herbivore recognition mechanisms

Perception of herbivorous insects was thus a very important development in plant defense signaling, and co-evolution between plants and insects has resulted in mechanisms that plants may exploit to recognize insect herbivores. This recognition can be mediated by the detection of sequential wounding events: Mithöfer *et al.* (2005) have shown that repeatedly inflicting damage on a plant elicits a stronger volatile response then a single wounding event. However, it has also been shown that sequential wounding alone is not sufficient to recreate the volatile response elicited by a feeding herbivore. A second mechanism by which plants can perceive herbivores is the recognition of specific components in the herbivore's oral secretions (OS). Recognition of herbivores via their OS has the advantage that it enables species-specific recognition: Diezel et al (2009) have shown that different herbivores elicit different responses from the plant, indicating that plants tailor their response to different herbivores. Relying on compounds present in insect OS creates selection pressure favoring insects that do not produce these compounds anymore. Therefore, there has most likely been strong selection for elicitors that are essential for the insect. The first insect elicitor to be isolated was the fatty-acid amino-acid conjugate (FAC) volicitin (17-OH-18:3-Gln), which was found in the OS of *Spodoptora exigua* (*S. exigua*) larvae feeding on *Zea mays* plants and shown to induce a volatile blend different from that induced by wounding alone (1997). Recently, volicitin was shown to play an important role in the caterpillar's nitrogen metabolism, showing that plants have evolved to recognize what may be an essential compound from a herbivore's OS (Yoshinaga *et al.*, 2008). Insect OS is not only used as recognition mechanism by plants, insects have also developed mechanisms to hamper the plant's defense response: The enzyme glucose oxidase, identified from *Helicoverpa zea* OS, was shown to counteract the production of nicotine induced by the feeding caterpillar (Musser *et al.*, 2002).

A number of other insect elicitors originating from various insects have been identified (Schmelz *et al.*, 2006a). FACs have also been shown to mediate most of the herbivore-specific responses of the coyote tobacco *Nicotiana attenuata* to the the Solanaceous specialist *Manduca sexta* (*M. sexta*) (Halitschke *et al.*, 2003). *M. sexta*'s FACs, are composed predominantly of linoleic acid (18:2) or linolenic acid (18:3) conjugated to Glu or Gln (Halitschke *et al.*, 2001).

In contrast to elicitors derived from plant pathogens, insect elicitor perception and mode of action is poorly understood; some FACs have been proposed to increase ion permeability in membrane bilayers, while in maize it has been shown that volicitin binds to a membrane-associated protein, suggesting a ligand-receptor interaction(Truitt *et al.*, 2004). What is completely unknown is the fate of insect elicitors when they are deposited on the wounded leaf tissue; an hypothesis was that

18:3-Glu could be cleaved into 18:3 and Glu, where the 18:3 could act as a substrate for jasmonate biosynthesis. In Chapter 2 I set out to investigate the metabolism of 18:3-Glu on wounded *N. attenuata* plants, while in Chapter 3 we discuss the relevance of FAC signaling in the early signaling events that occur in *N. attenuata*.

Heterogeneity in the jasmonate response

After the plants perceives wounding and subsequent deposition of oral secretions by an insect herbivore, jasmonate biosynthesis is activated, and this jasmonate accumulation, along with its perception, is crucial for defense against herbivores (Kessler *et al.*, 2004; McConn *et al.*, 1997). Jasmonate biosynthesis commences in the chloroplast with the lipase-mediate release of linolenic acid (18:3), which is subsequently oxygenized to 13*S*-hydroperoxy-18:3, a step catalyzed by 13-lipoxygenases (LOXs). The plastidial enzymes allene oxide synthase (AOS) and allene oxide cyclase (AOC) convert the LOX product into (9*S*,13*S*)-12-oxo-phytodienoic acid (OPDA) which is then transported into the peroxisome where it is reduced by an OPDA reductase (OPR3) and β-oxidized to generate (3*R*,7*S*)-jasmonic acid [reviewed by Wasternack, (2007)].

A common feature of plant hormones is their chemical modification to moderate activity, and JA can be modified in a variety of ways: It can be conjugated to amino acids, sugars, or methyl groups on its carboxyl group; and it can be hydroxylated at the 11- or 12-position, and these hydroxylated forms can subsequently be glucosidated, or oxidized to a carboxylic acid. Combinations of these forms (e.g. a JA-amino acid conjugate with an OH group on the 12-position) are also possible. Figure 1 gives an overview of the numerous possibilities. Of these JA derivatives, JA-isoleucine (JA-Ile) has been shown to be active role in the initiation of the defense response; JA-Ile is biosynthesized from JA by JASMONATE RESISTANT-1, (JAR1) in *A. thaliana* (Staswick and Tiryaki, 2004) and JAR4 and JAR6 in *N. attenuata* (Wang *et al.*, 2007). Studies in *A. thaliana* have shown that JA-Ile is necessary for the interaction between the F-box

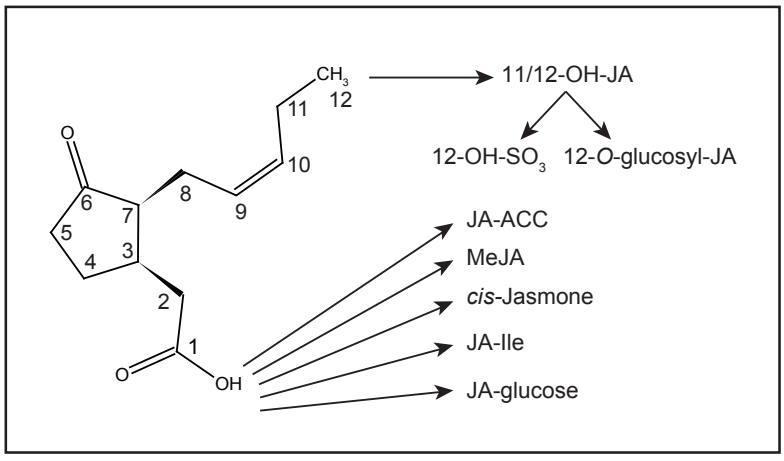

Figure 1 Possible modification of jasmonic acid.
Metabolism of JA can take place at the carboxylic acid group to form a variety of additions and reductions, while the C11 and C12 can by oxidized to OH groups. 12-OH-JA can be further metabolized to sulfonated and glycosilated forms.

protein CORONATINE INSENSITIVE 1 (COI1) and JASMONATE ZIM DOMAIN 1 (JAZ1)(Chini *et al.*, 2007; Thines *et al.*, 2007), the first step in initiating many jasmonate-mediated transcriptional responses.

COI1 is an essential component for intact jasmonate signaling, *A. thaliana coi1* mutants, *Solanum lycopersicum jai1* mutants (mutated in the COI1 gene) and *N. attenuata* ir*coi1* plants with decreased transcription of NaCOI1 all showed a reduced resistance to a plethora of herbivores, both in the glasshouse and in the field (Li *et al.*, 2004; Paschold *et al.*, 2007; Xie *et al.*, 1998). Although JA-Ile has been demonstrated for the COI1/JAZ1 complex to function (Katsir *et al.*, 2008), the phenotype of *jar1 A. thaliana* plants is still substantially different from *coi1* plants, there is one report showing that *jar1* plants have less resistance to fungi (Staswick *et al.*, 1998), but lack reduced induced defenses to the extend described for *coi1* plants. A hypothesis for this discrepancy is that *jar1* plants are 'leaky', and still produce ~10% of WT JA-Ile levels (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). An alternative hypothesis is that there are other active jasmonates that can active defense responses. However, JA-Ile is so far the only jasmonate that has been shown to bind with high affinity to a JAZ/COI1 complex.

Although a plethora of jasmonates have been identified, only a few have been functionally characterized. These are JA-Ile's described properties, 12-OH-JA which induces tuber formation in *Solanum tuberosum* plants (Koda and Okazawa, 1988), and which may negatively regulate JA biosynthesis (Miersch *et al.*, 2008). It has been proposed that in *N. attenuata*, COI1 regulates JA biosynthesis by mediating gene transcription, and JA-Ile by affecting JA-Ile turnover (Paschold *et al.*, 2008). However, little is know about the regulation of other jasmonates. Moreover, the accumulation of jasmonates is variable in different plant species: JA and JA-Ile are still substantially induced 24h after wounding *A. thaliana* plants (Glauser *et al.*, 2008), while in *N. attenuata* their accumulation is transient, and drops back to basal levels after 2 to 3h (Halitschke and Baldwin, 2003). In *S. lycopersicum*, 12-OH-JA accumulated to different concentrations in two different cultivars, showing that even within the same plant species there can be considerable variation (Miersch *et al.*, 2008). Different wild populations of *N. attenuata* also showed a high variety in their jasmonate accumulation patterns (Schuman *et al.*, 2009).

The gaps in our knowledge about the regulation of jasmonate metabolism and the different accumulation patterns motivated us to study jasmonate signaling in a relatively uncharacterized Solanaceous species, *Solanum nigrum*. As tools for this study, I used inverted-repeat transgenic lines silenced in a 13-lipoxygenase to reduce JA biosynthesis; plants silenced in the enzyme JAR4 which

conjugates JA to JA-Ile; and plants silenced for the jasmonate receptor COI1 to study jasmonate perception. In order to study the temporal effects of the local JA burst, I looked at the activation of a systemic defense response. From previous work on this wild species we know that it shares only about 10% of its transcriptional response to *M. sexta* feeding with the Solanaceous plant *N. attenuata* (Schmidt *et al.*, 2005). In Chapter 4 I describe the jasmonate kinetics in the different silenced lines, and show that both SnCOI1 and SnJAR4 play important, but only partly overlapping roles in JA metabolism and the activation of systemic signaling.

Consequences of jasmonate signaling on a transcriptomic, metabolomic and ecological level

'Plant defense' is a simple term for a very complex system of defenses and re-allocation of resources. Most of these changes are preceded by transcriptional activation, and many different plant species respond transcriptionally to herbivory or wounding (De Vos *et al.*, 2005). These transcriptomic changes lead to a metabolic re-arrangement, where the plant may accumulate toxic or deterring metabolites and produce indirect defenses that can attract the predators of herbivores. Microarrays can analyze gene expression of many genes at once, and are useful tools to analyze large-scale gene expression, and can reveal patterns that are harder to elucidate in smaller-scale experiments. For example, the transcriptional response of *A. thaliana* to aphids feeding is completely different than from caterpillar feeding, demonstrating that herbivores from different feeding guilds elicit different responses in this plant species (De Vos *et al.*, 2005). Microarray experiment also confirmed that FACs from *M. sexta*'s OS elicit a highly similar response to that elicited by caterpillar feeding in *N. attenuata* (Halitschke *et al.*, 2003). The annotation of microarrays is improving with the advance of new sequencing techniques, and genes can be grouped together using a gene identifier, which gives more information about the class a certain gene belongs to.

Monitoring changes in the metabolome has become easier with the development of ultra-high pressure liquid chromatography coupled to high-resolution mass spectrometry (UPLC-MS). This technique allows for short (15 min.) runs that can detect up to 2,000 compounds with a mass accuracy of 3-5ppm. This technique is unbiased in the sense that no previous information about the sample or the components is required, but it still selects against compounds that do not ionize in the source chosen for the mass spectrometer. Although the resolution of the last-generation mass spectrometers is approaching the 1 to 3ppm range, annotation of ions is still relatively poor; resolving molecular formulas of larger ions requires extreme mass precision. Therefore, the structural elucidation of unknown ions is still a bottleneck in metabolomics.

Changes in transcription and metabolism produce a "better defended" plant, which should better resist or tolerate herbivores. To measure how much "better" the plant is defended, one can

"shake a reagent grade herbivore" (Kant and Baldwin, 2007), on the plant and measure its weight or the amount of plant damage. However, depending on the herbivore used, these experiments might not reflect field condition in which a multitude of conditions influence herbivore performance. Field experiments are therefore a vital tool for testing hypotheses about what contributes to a "better-defended" plant, and what does not. In Chapter 5, I was interested to see how the previously described ir*lox3*, ir*jar4* and ir*coi1* plants respond to local insect damage transcriptionally and metabolically in comparison to WT plants, and how this data would correspond to damage levels in field experiments.

Scope of this thesis

In plant defense, there are four important "phases": First, the plant recognizes the feeding herbivore; second, the plant activates hormone signaling; third, these hormones will activate transcripts of genes that will lead to, fourth, a metabolic re-arrangement that will finally result in a "defended plant". In this thesis, I have explored each of these areas, and have addressed the following questions:

- (Chapter 2) Is the FAC 18:3-Glu metabolized on the leaf surface, and if yes, what products occur, and what is the function of these products?
- (Chapter 3) What are the functions of FACs in the initiation of the defense response?
- (Chapter 4) What is the role of jasmonate biosynthesis, conjugation and perception in the accumulation patterns of JA and its derivatives in *Solanum nigrum*, how is the metabolism of JA regulated, and what is the effect of local jasmonate accumulation on the activation of systemic defense responses?
- (Chapter 5) What role does jasmonate conjugation and perception play in the activation of short-term gene transcription and rearrangement of plant metabolism and what is the impact on the plant in a natural environment?

Overview of Manuscripts

Manuscript I

Rapid modification of the insect elicitor *N*-linolenoyl-glutamate via a lipoxygenase-mediated mechanism on *Nicotiana attenuata* leaves

Arjen van Doorn, Mario Kallenbach, Alejandro A. Borquez, Ian T. Baldwin and Gustavo Bonaventure

Published in *BMC Plant Biology* 10 (164)

In manuscript I, we describe the metabolism of the the insect elicitor *N*-linolenoyl-glutamate (18:3-Glu) on the wounded leaf surface, and show that 18:3-Glu is metabolized into at least three different 18:3-Glu derivatives. One of these derivatives was shown to be active as an elicitor of jasmonic acid and monoterpenes.

A. van Doorn and G. Bonaventure planned and performed the experiments, analyzed data and wrote the manuscript. A.A. Borquez performed experiments and M. Kallenbach interpreted the MS/MS spectra and was involved in structural elucidation. I.T. Baldwin was involved in planning the experiments and correcting the manuscript.

Manuscript II

Herbivore associated molecular patterns: FAC signaling and metabolism

Gustavo Bonaventure, Arjen van Doorn and Ian T. Baldwin

Provisionally accepted for publication in *TRENDS in plant science*

In Manuscript II, we review the literature regarding FAC signaling and metabolism, using recent advances in the model system of *Nicotiana attenuata* and *Manduca sexta*.

G. Bonaventure drafted the manuscript. A. van Doorn commented on the manuscript and prepared the figures. I.T. Baldwin was involved in the planning and correction of the manuscript.

Manuscript III

Regulation of jasmonate metabolism and activation of systemic signaling in *Solanum nigrum*: COI1 and JAR4 play overlapping yet distinct roles

Arjen van Doorn, Gustavo Bonaventure, Dominik D. Schmidt and Ian T. Baldwin

Accepted for publication in *New Phytologist*

Manuscript III describes the characterization of the jasmonate acid (JA) metabolism and regulation in *Solanum nigrum*. Three genes in the jasmonate signaling cascade were silenced, and this way we showed that JA metabolism is depending on SnCOI1. We also showed that local JA accumulation correlated strongly with the activation of systemic defenses.

A. van Doorn planned and performed the experiments, analyzed the data and drafted the manuscript. G. Bonaventure analyzed data and corrected the manuscript. D. D. Schmidt was involved in planning and performing the experiments. I.T. Baldwin conceived of and directed the study, and corrected the manuscript.

Manuscript IV

JA-Ile signaling is not required for defense responses in nature

Arjen van Doorn, Gustavo Bonaventure, Ilana Rogachev, Asaph Aharoni and Ian T. Baldwin.

In preparation

In manuscript IV we profiled the transcriptional and metabolomic changes following herbivory, and the influence of jasmonate signaling on these induced changes. Moreover, we studied the performance of plant silenced in jasmonate biosynthesis, conjugation to JA-Ile and perception in the field.

A. van Doorn performed experiments, analyzed data and drafted the manuscript. G. Bonaventure was involved in planning the experiments, data analysis and corrected the manuscript. I. Rogachev and A. Aharoni were involved in performing the LC-TOF measurements and interpretation of MS/MS spectra. I.T. Baldwin was involved in planning and performing the field experiments, conceived and directed the study, and corrected the manuscript.

Chapter 2

Rapid Modification of the Insect Elicitor *N*-linolenoyl-glutamate via a Lipoxygenase-mediated Mechanism on *Nicotiana attenuata* Leaves

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Rapid modification of the insect elicitor N-linolenoyl-glutamate via a lipoxygenase-mediated mechanism on *Nicotiana attenuata* leaves

Arjen VanDoorn, Mario Kallenbach, Alejandro A Borquez, Ian T Baldwin, Gustavo Bonaventure*

Abstract

Background: Some plants distinguish mechanical wounding from herbivore attack by recognizing specific constituents of larval oral secretions (OS) which are introduced into plant wounds during feeding. Fatty acid-amino acid conjugates (FACs) are major constituents of *Manduca sexta* OS and strong elicitors of herbivore-induced defense responses in *Nicotiana attenuata* plants.

Results: The metabolism of one of the major FACs in *M. sexta* OS, *N*-linolenoyl-glutamic acid (18:3-Glu), was analyzed on *N. attenuata* wounded leaf surfaces. Between 50 to 70% of the 18:3-Glu in the OS or of synthetic 18:3-Glu were metabolized within 30 seconds of application to leaf wounds. This heat-labile process did not result in free α-linolenic acid (18:3) and glutamate but in the biogenesis of metabolites both more and less polar than 18:3-Glu. Identification of the major modified forms of this FAC showed that they corresponded to 13-hydroxy-18:3-Glu, 13-hydroperoxy-18:3-Glu and 13-oxo-13:2-Glu. The formation of these metabolites occurred on the wounded leaf surface and it was dependent on lipoxygenase (LOX) activity; plants silenced in the expression of *NaLOX2* and *NaLOX3* genes showed more than 50% reduced rates of 18:3-Glu conversion and accumulated smaller amounts of the oxygenated derivatives compared to wild-type plants. Similar to 18:3-Glu, 13-oxo-13:2-Glu activated the enhanced accumulation of jasmonic acid (JA) in *N. attenuata* leaves whereas 13-hydroxy-18:3-Glu did not. Moreover, compared to 18:3-Glu elicitation, 13-oxo-13:2-Glu induced the differential emission of two monoterpene volatiles (β-pinene and an unidentified monoterpene) in ir*lox2* plants.

Conclusions: The metabolism of one of the major elicitors of herbivore-specific responses in *N. attenuata* plants, 18:3-Glu, results in the formation of oxidized forms of this FAC by a LOX-dependent mechanism. One of these derivatives, 13-oxo-13:2-Glu, is an active elicitor of JA biosynthesis and differential monoterpene emission.

Background

Interactions between plants and invertebrate herbivores have a long history; the first evidence of plant damage by arthropods dates back 400 m years ago [1]. This timeframe has allowed plants and insects to develop sophisticated mechanisms to recognize one another and respond accordingly. Plants activate a plethora of defense responses upon insect feeding, and one way of decreasing the herbivore load is to emit volatiles that attract predators or parasitoids of the herbivore [2,3]. These herbivore-induced plant volatiles (HIPVs) consist of different compounds, for example C_6 green leaf volatiles (GLVs) and isoprenoids such as C_{10} monoterpenes. The plant's ability to produce different volatile signals when attacked by herbivores is essential for the function of these molecules as indirect defenses. Depending on the plant species, the recognition of insect feeding may be primarily mediated by mechanisms such as the perception of components in insect oral secretions (OS) [4-6], multiple sequential wounding events that mimic larvae feeding [7], or a combination of both.

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Recently, the discovery of digested fragments of a plant ATP synthase, named inceptins, as elicitors of insect responses added a plant 'self-recognition' mechanism to the repertoire of mechanism for insect's feeding perception [8,9]. Among the insect elicitors of plant defense responses, the first to be isolated was the fatty-acid amino-acid conjugate (FAC) volicitin (17-OH-18:3-Gln), which was found in the OS of *Spodoptora exigua* (*S. exigua*) larvae feeding on maize (*Zea mays*) plants and shown to induce a volatile blend different from that induced by wounding alone [6]. Glucose oxidase was first identified from the corn earworm, *Helicoverpa zea;* [10] and it has been demonstrated to suppress the plant's defense response [11] and activate the salicylic acid (SA) pathway [12]. Inceptins were found in the OS of *Spodoptora frudgiperda* larvae feeding on cowpea (*Vigna unguiculata*) and have the capacity to induce the differential production of jasmonic acid (JA), SA and volatiles in cowpea plants [9]. More recently, sulfur-containing compounds, named caeliferins, were isolated from grasshopper OS (*Schistocerca americana*) and were able to induce volatile production in maize plants [13]. Finally, in a recent study, different elicitors were applied on a variety of plant species, and phytohormones and volatile production were monitored. The results indicated that elicitation by different insect-derived components is a plant-species specific process [5].

M. sexta's main elicitors to induce insect specific defense responses in *Nicotiana attenuata* plants are FACs, which are composed predominantly of linoleic acid (18:2) or linolenic acid (18:3) conjugated to Glu or Gln [14]. When applied to wounded *N. attenuata* leaves, synthetic FACs induce the differential production of jasmonic acid (JA) and ethylene [14,15], large scale transcriptomic and proteomic changes [4,16], and the release of HIPVs [17]. Moreover, when removed from *M. sexta* OS, the remaining FAC-free OS fraction loses its capacity to elicit insect specific responses in *N. attenuata* [4,16,17] which can be recovered after reconstitution of the FAC-free OS fraction with synthetic FACs [4]. The long-standing question of why a caterpillar would produce these potent elicitors was addressed in a recent study demonstrating the essential role of Gln containing FACs in nitrogen assimilation by *Spodoptora litura* larvae [18].

In contrast to elicitors derived from plant pathogens, insect elicitor perception and mode of action is poorly understood. It is known that in maize, volicitin binds to a membrane-associated protein suggesting a ligand-receptor interaction [19]. Additional proposed mechanisms for FAC elicitation include their capacity to increase ion permeability in membrane bilayers [20]. It has been previously demonstrated that volicitin can be transferred from the caterpillar OS into the wound surfaces of maize leaves [21] and although the transferred amounts may be low [22], they seem sufficient to elicit specific responses against insect herbivores [23].

To understand the metabolic fate of FACs in plants and to gain novel insights into their mode of action, we investigated the metabolism of one of the major FACs in *M. sexta* OS, 18:3-Glu, on *N. attenuata* leaves. We studied the consequences of its metabolism on two processes associated to herbivory, the regulation of JA biosynthesis and terpenoid volatile emission.

Results

N-linolenoyl-glutamate is rapidly metabolized on wounded *N. attenuata* leaf surfaces

To investigate the turnover rate of 18:3-Glu on wounded *N. attenuata* leaves, 0.17 nmoles of synthetic 18:3-Glu, the amount naturally occurring in 10 μL *M. sexta* OS [14], were applied onto puncture wounds. Leaf material was harvested at different time points and 18:3-Glu levels were quantified by LC-MS/MS. The results showed that 18:3-Glu levels decreased rapidly, with 70% of the initial 18:3-Glu being metabolized within 30 seconds (Fig. 1a). To assess whether the endogenous 18:3-Glu in the insect's OS was also rapidly metabolized, 10 μL of *M. sexta* OS were applied on wounded leaf tissue and the 18:3-Glu levels were analyzed. The results again revealed a rapid decline of the 18:3-Glu levels in the OS with 50% of the initial 18:3-Glu metabolized within 30 seconds (Fig. 1b).

The metabolic fate of 18:3-Glu on the wounded leaf surface was investigated by applying both, synthetic radiolabeled [1-^{14}C]18:3-Glu and *M. sexta* OS spiked with [1-^{14}C]18:3-Glu, onto wounded leaf tissue for 2 min. After extraction and chromatographic separation of the ^{14}C-labeled metabolites by thin layer chromatography (TLC), the results showed that 18:3-Glu was not hydrolyzed into free 18:3 and Glu, but converted into different 18:3-Glu modified forms (Fig. 2a, Additional file 1). As evaluated by their Rfs, the major modified compounds were more polar than the unmodified 18:3-Glu, however, minor (less polar modified forms) were also detected (Additional file 1). Consistent with the rapid metabolism of unlabeled 18:3-Glu, radiolabeled 18:3-Glu derivatives appeared within the first minutes upon contact with wounded leaf tissue (Additional file 1). The ^{14}C-labeled metabolites were also separated and quantified by reverse phase (RP) radio-HPLC, which detected three major peaks with retention times consistent with an increased polarity compared to 18:3-Glu (Fig. 2a and 2b). The total radioactivity recovered after extraction was ca. 90% of the initial radioactivity applied onto the leaf surface, and the peak areas corresponding to metabolites 1, 2, 3 and to the unmodified 18:3-Glu (Fig. 2a) accounted for ca. 8, 15, 35 and 30% of the recovered radioactivity, respectively. These results were

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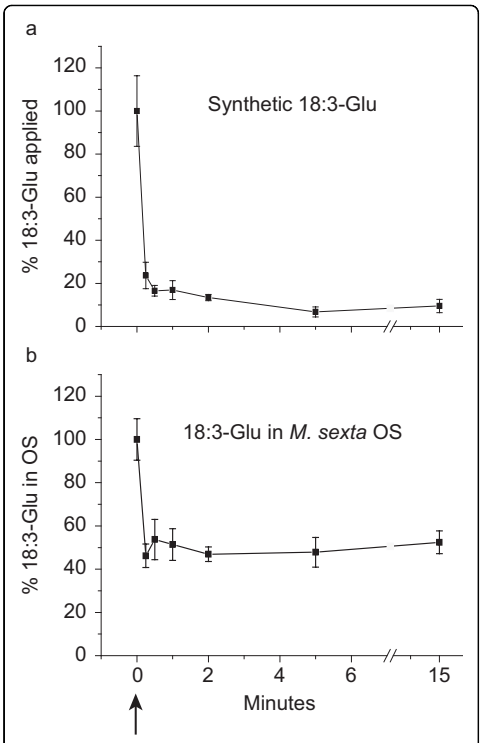

Figure 1 18:3-Glu is rapidly metabolized on wounds of *N. attenuata* leaves. Three lines of puncture wounds were generated on leaves with a fabric pattern wheel and **(a)** 0.17 nmoles of synthetic 18:3-Glu, or **(b)** 10 μL *M. sexta* OS (containing approximately 0.17 nmoles of 18:3-Glu) were applied onto the wounds. Leaves were extracted at different times and 18:3-Glu levels quantified by LC-MS/MS. Initial 18:3-Glu amounts (T0 ↑) were set at 100%. Bars represent standard errors (± SE, n = 4).

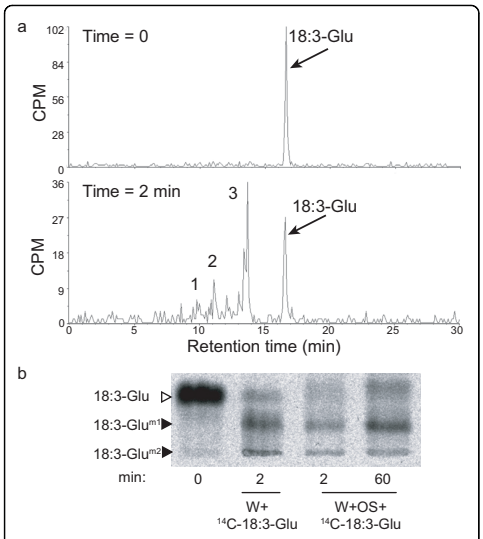

Figure 2 Radio-HPLC chromatograms of wounded *N. attenuata* leaves treated with ^{14}C-18:3-Glu. **(a)** Leaves of rosette stage *N. attenuata* plants were wounded with a pattern wheel and 0.1 μCi of [1-^{14}C]18:3-Glu were immediately applied onto leaf wounds. The damaged tissue was harvested immediately (T0) or 2 min after the treatment. After extraction, the radiolabeled metabolites were separated by RP-radio-HPLC. The numbers (1 to 3) indicate the different fractions collected for further analysis. CPM: counts per minute. (**b**) *M. sexta* OS was spiked with 0.1 μCi ^{14}C 18:3-Glu, applied onto wounded leaf tissue, extracted after different time points and separated by TLC (W+OS). Plates were exposed for 24 h to ^{14}C-sensitive screens. Time 0: ^{14}C 18:3-Glu was applied onto wounded leaves and immediately extracted. W+: ^{14}C-18:3-Glu was applied onto wounded leaves and extracted after 2 min. 18:3-Glu^{m1} and 18:3-Glu^{m2} indicate different modified forms of 18:3-Glu.

consistent with the rate of 18:3-Glu metabolism shown in Fig. 1 and indicated that the metabolites corresponding to peaks 1, 2 and 3 in Fig. 2a were the major metabolites produced.

Isolation and chemical characterization of 18:3-Glu derivatives

For the purification of the three major polar 18:3-Glu derivatives, a leaf extract derived from wounded 18:3-Glu-treated *N. attenuata* leaves was fractionated by HPLC as described in Materials and Methods. Based on the retention times of the radiolabeled forms of 18:3-Glu, three HPLC fractions were collected and analyzed by LC-ESI-ToF to identify candidate ions corresponding to modified forms of 18:3-Glu. After mass/charge based selection (600 >*m/z* > 200) and retention times, three compounds corresponding to the major ions *m/z* 352.1779, 422.2549 and 438.2550 in fractions 1, 2 and 3 (Fig. 2a), respectively, were selected and further purified by preparative TLC (see Material and Methods). These compounds were used for all subsequent analyses. To confirm that these compounds were derivatives of 18:3-Glu, they were directly injected into a triple-quad ESI-MS/MS system. After fragmentation of their molecular ions by collision induced dissociation (CID), all compounds released an intense ion with *m/z* 128, a specific ion generated from the rearrangement of the Glu moiety [24]. Additionally, fragmentation of the ions with *m/z* 422.3 and 438.3 using increasing fragmentation energies clearly showed an energy-dependent neutral loss of water from both ions. However, the ion with

m/z 438.3 lost water already with a fragmentation energy of 10V while the ion with *m/z* 422.3 with 15.5V, suggesting the presence of a more labile oxygenated functional group in the ion with *m/z* 438.3.

For structure elucidation, MS/MS was performed using an LC-ESI-XL-orbitrap. Consistent with the triple-quad ESI-MS/MS analysis described above, all components released an intense ion with *m/z* 128.0368 [24]. For the compound purified from fraction 1 (Fig. 3a), an intense ion with *m/z* 223.1359 was released after fragmentation. This ion was generated from the acyl chain of the modified 18:3-Glu including the re-arrangement described in [24]. From this acyl fragment, a sub-fragment of *m/z* 195.1409 was released, giving a difference of 27.995 Da, which was consistent with the release of CO from the ω-position (Fig. 3a). The spectrum was consistent with compound 1 being 13-oxo-trideca-9,11-dienoyl-Glu. (13-oxo-13:2-Glu). For the compound purified from fraction 2 (Fig. 3b), an intense ion with *m/z* 404.2485 was detected and corresponded to the neutral loss of water from the molecular ion (*m/z* 422.2549), consistent with the triplequad ESI-MS/MS analysis described above. A second intense ion with *m/z* 293.2153 originated from the acyl chain of the modified 18:3-Glu including the re-arrangement described in [24] and the addition of a hydroxyl group (Fig. 3b). This fragment showed a subsequent neutral loss of 69.0708 Da giving *m/z* 224.1445, indicating the loss of C_5H_9 (calculated mass 69.0705 Da) from the end of the acyl chain (Fig. 3b) and the position of the hydroxyl group at C_{13} of the 18:3 moiety. Further evidence for the position of the hydroxyl group was the loss of 98.0739 from the *m/z* 293.2147 to give *m/z* 195.1408, corresponding to a loss of $C_6H_{10}O$ (calculated mass 98.0732 Da). The spectrum was consistent with compound 2 being 13-OH-octodeca-9,11,15-trienoyl-Glu (13-OH-18:3-Glu). For the compound purified from fraction 3 (Fig. 3c), an intense ion with *m/z* 420.2431 was detected and corresponded to the neutral loss of water from the molecular ion (*m/z* 438.2550), again consistent with the triplequad ESI-MS/MS analysis described above. A fragment with *m/z* 291.1996, generated from the acyl chain of the modified 18:3-Glu and including the re-arrangement described in [24] was consistent with the previous loss of water from a hydroperoxy group. Moreover, a fragment ion with *m/z* 352.1799 was also generated, which was identical to the molecular ion of 13-oxo-13:2-Glu and suggested the generation of this compound from a 13-hydroperoxy-dated precursor in the ion source. The proposed structure for fraction 3 was 13-OOH-octodeca-9,11,15-trienoyl-Glu (13-OOH-18:3-Glu).

Kinetic of formation of 18:3-Glu oxidized forms in wounded *N. attenuata* leaves

The formation of 18:3-Glu-modified forms on wounded *N. attenuata* leaves was analyzed after different times. Synthetic 18:3-Glu (0.17 nmoles) were applied on puncture wounds and after extraction samples were analyzed by LC-MS/MS and the results are presented in Fig. 4. Because ionization efficiencies vary substantially between compounds, the amounts of the different compounds are presented as normalized peak areas. Consistent with the rapid kinetic of 18:3-Glu metabolism (Fig. 1 and 2), the oxidized forms of 18:3-Glu were generated rapidly (Fig. 4). All three compounds, 13-OH-18:3-Glu, 13-OOH-18:3-Glu and 13-oxo-13:2-Glu started to accumulate within 15 seconds. 13-OH-18:3-Glu was the most abundant derivative and peaked at 15 seconds, while 13-OOH-18:3-Glu and 13-oxo-13:2-Glu showed a slower kinetic of accumulation and their relative levels were lower (Fig. 4). The relative levels of these oxygenated derivatives detected by LC-MS/MS (2 > 1 > 3; Fig. 4) differed from those detected by radio-HPLC (3 > 2 > 1; Fig. 2b) and, as mentioned above, these differences most likely reflected variations in ionization efficiencies between derivatives. The detection of 13-OH-18:3-Glu at time zero indicated that conversion of 18:3-Glu already occurred after a few seconds of its contact with wounded tissue (the time required to harvest the leaf after application of 18:3-Glu, ~3-5 seconds).

Formation of 13-oxo-18:3-Glu occurs on the leaf surface

It has been reported that formation of 13-oxo-trideca-dienoic acid (13-oxo-13:2) from 13-OOH-linolenic acid (13-OOH-18:3) could occur by both enzymatic [25] and non-enzymatic mechanisms (thermal decomposition; [25,26]). To rule out the possibility that 13-oxo-13:2-Glu was generated by thermal decomposition during sample preparation or analysis (for example during solvent evaporation or electrospray ionization), wounded leaves supplemented with synthetic 18:3-Glu were extracted with and without the addition of 1% butylhydroxytoluene (BHT) as a radical scavenger and of 10 mg/ml of trimethylphosphite (TMP) as a reducing agent of the hydroperoxy groups [27] in the solvent. Samples were taken after 1 and 5 min of the treatment and extractions and solvent evaporation were conducted always on ice to prevent sample heating. After analysis by LC-MS/MS, the results showed that formation of 13-oxo-13:2-Glu was independent of the presence of BHT and TMP in the extraction solvents (Additional file 2) and demonstrated that its biogenesis took place on the leaf surface.

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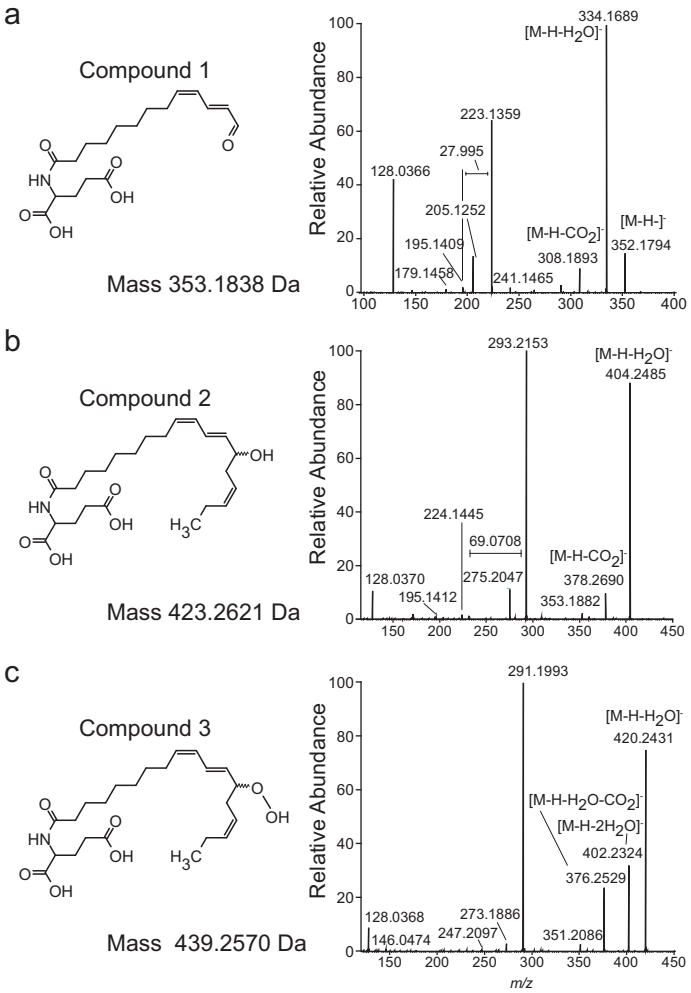

Figure 3 MS analysis of the purified 18:3-Glu derivatives. **(a, b, c)**. Proposed structures and MS spectra obtained by LC-ESI-XL-Orbitrap analysis (negative mode) of the three 18:3-Glu derivatives purified from fractions 1, 2 and 3 in Fig. 2. See text for a detailed discussion of the MS spectra.

Oxidation of 18:3-Glu on wounded *N. attenuata* leaves depends on lipoxygenase activity

To test if the metabolism of 18:3-Glu was enzymatic, steamed wounded leaves were supplemented with 18:3-Glu and its turnover rate and modifications determined by LC-MS/MS. In this experiment, control leaves (18:3-Glu control) were freeze killed before application of 18:3-Glu onto their frozen surface and 18:3-Glu levels represent the initial amounts applied. Consistent with the abovementioned results, more than 90% of the applied 18:3-Glu was metabolized after 5 min of the treatment in wounded leaves (Fig. 5a). In contrast, after steam treatment of leaves to inactivate heat-labile processes, the levels of 18:3-Glu remained higher compared

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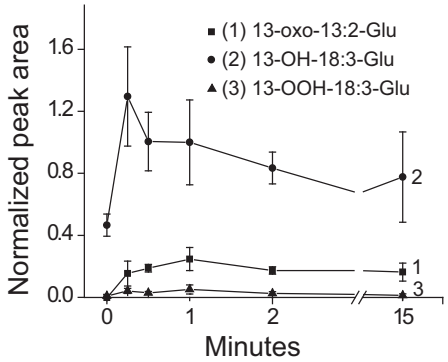

Figure 4 Accumulation of 18:3-Glu derivatives on wounded WT *N. attenuata* leaves. WT plants were wounded with a pattern wheel and 0.17 nmoles of 18:3-Glu were applied to the wounds and samples harvested after different times. After extraction, samples were analyzed by LC-MS/MS (n = 3, bars denote ± SE). Numbers between brackets correspond to compounds 1, 2 and 3 in Fig. 2 and 3.

to wounded leaves and statistically similar to control levels (Fig. 5a). Consistently, the formation of 18:3-Glu derivatives was not detected in steamed leaves (data not shown). In the absence of wounding, the levels of 18:3-Glu were also not reduced compared to the control treatment (Fig. 5a). Together, these results indicated that the metabolism of 18:3-Glu was primarily enzymatic and that it required mechanical damage of the leaf.

Based on the identification of a 13-hydroperoxide derivative, we hypothesized that lipoxygenase activity was responsible for hydroperoxidation of 18:3-Glu. *N. attenuata* leaves express two major plastidial lipoxygenases, lipoxygenase 2 (NaLOX2) and lipoxygenase 3 (NaLOX3), involved in the supply of hydroperoxy-fatty acids for green leaf volatiles and JA biosynthesis, respectively [28,29]. Hence, we tested 18:3-Glu metabolism in *N. attenuata* plants silenced in the expression of either *NaLOX2* (ir*lox2*) or *NaLOX3* (ir*lox3*). The expression of *NaLOX2* and *NaLOX3* transcripts is reduced by 99% and 83% in ir*lox2* and ir*lox3* plants, respectively [29]. Importantly, the transcript levels of NaLOX3 are also reduced (by 94%) in ir*lox2* plants, most likely due to co-silencing [29]. In contrast, the transcript levels of NaLOX3 in ir*lox2* plants were similar to WT [29]. The accumulation of 18:3-Glu derivatives after 18:3-Glu treatment was first analyzed in these plants (Fig. 5b). All genotypes were substantially reduced in their ability to produce 18:3-Glu derivatives with ir*lox2* plants showing the strongest reduction in

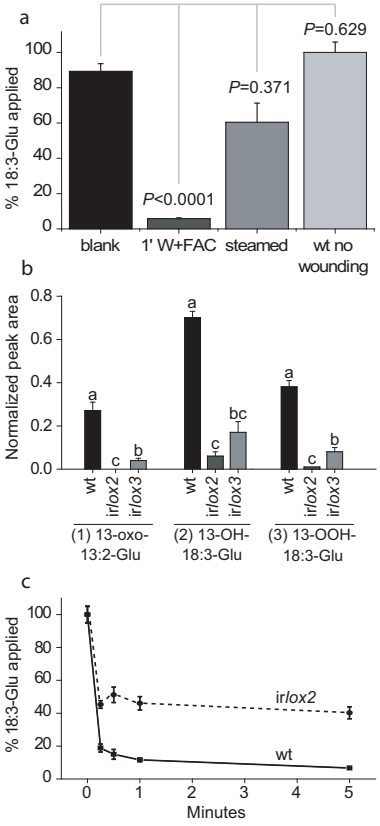

Figure 5 Analysis of 18:3-Glu metabolism in steamed leaves and in LOX deficient plants. **(a)** 18:3-Glu (0.17 nmoles) was applied to either wounded leaves, wounded-steamed leaves, or unwounded leaves. The 18:3-Glu blank sample denotes an unwounded leaf onto which 18:3-Glu was added after freeze killing the leaf. Leaves were extracted after 5 min of the treatments and 18:3-Glu levels were analyzed by LC-MS/MS. Statistical analysis was performed using a non-parametric Moses extreme test with Bonferroni correction, * indicates $P < 0.017$, N.S. denotes non-significant differences (n = 3, bars denote ± SE). **(b)** Leaves of ir*lox2* and ir*lox3* plants were wounded, 0.17 nmoles of 18:3-Glu were applied on the wounds, and the samples were harvested after 2 min. After extraction, 18:3-Glu derivatives were analyzed by LC-MS/MS (n = 3, bars denote ± SE). Different letters indicate significant differences (univariate *ANOVA* 13-oxo-13:2-Glu: $F_{3,3} = 85.4$ $P < 0.001$, 13-OH-18:3-Glu: $F_{3,3} = 25.1$ $P < 0.001$ 13-OH-18:3-Glu: $F_{3,3} = 136.1$ $P < 0.001$ followed by a Scheffé *post-hoc* test, $P < 0.05$). **(c)** Turnover of 18:3-Glu on WT and ir*lox2* plants. Leaves were wounded, 18:3-Glu (0.17 nmoles) were applied onto the wounds, and the samples were harvested after different times. 18:3-Glu levels were analyzed by LC-MS/MS. Initial 18:3-Glu amounts (T0) were set at 100% (n = 4, bars denote ± SE).

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their accumulation. The rate of 18:3-Glu turnover was further analyzed in ir*lox2* plants and, consistent with the reduced accumulation of oxygenated forms of 18:3-Glu on wounded leaves of this genotype, the turnover rate was significantly reduced compared to wild-type plants (Fig. 5c). After 5 min, 40% of the applied amounts of 18:3-Glu remained on the wounded leaf surface compared to 7% on WT (Fig. 5c).

13-oxo-13:2-Glu is an active elicitor

FAC elicitation of leaves in *N. attenuata* plants induces an enhanced production (2 to 3-fold) of JA compared to mechanical damage and this response was used as a parameter to test elicitation activity [5,14]. For this experiment synthetic 18:3-Glu and purified 13-oxo-13:2-Glu and 13-OH-18:3-Glu were applied onto wounds of *N. attenuata* leaves and JA levels were quantified after 60 min of the treatments. This time point corresponds to the peak of JA accumulation in *N. attenuata* leaves after FAC elicitation [30]. For this and subsequent elicitation experiments, the amounts of elicitors applied corresponded to either 0.17 nmoles of synthetic 18:3-Glu or the corresponding amounts of 13-oxo-13:2-Glu and 13-OH-18:3-Glu produced by wounded leaves after 2 min (as assessed by LC-MS/MS; see Materials and Methods). The instability of the purified 13-OOH-18:3-Glu precluded its analysis as an elicitor. 13-oxo-13:2-Glu induced JA production to similar levels as 18:3-Glu and ~2-fold over wounding whereas 13-OH-18:3-Glu did not enhance JA production compared to wounding (Fig. 6a). Quantification of JA levels at 60, 90 and 120 min after 18:3-Glu and 13-oxo-13:2-Glu elicitation showed that both elicitors enhanced JA accumulation to similar levels at these 3 time points in both WT and ir*lox2* (data not shown).

The emission of terpenoid volatiles in *N. attenuata* plants is influenced by application of FACs to the wounds [17]. To investigate if the metabolism of 18:3-Glu could qualitatively or quantitatively affect terpenoid volatile emission, WT and ir*lox2* plants were analyzed after wounding and application of either 18:3-Glu or 13-oxo-13:2-Glu. Terpenoid volatiles in *N. attenuata* are released in a diurnal cycle [17] and for this experiment volatiles were trapped after 24 h of the treatments for a period of 8 h. The results showed that two monoterpenes, β-pinene and a monoterpene of unidentified structure (see Additional file 3 for MS spectra) were differentially emitted in ir*lox2* plants when 13-oxo-13:2-Glu and 18:3-Glu elicitation treatments were compared (Fig. 6b). In contrast, emission of these two monoterpenes in WT plants was similar between treatments (Fig. 6c). *trans*-α-bergamotene and an additional sesquiterpene did not show significant differences between the treatments (data not shown).

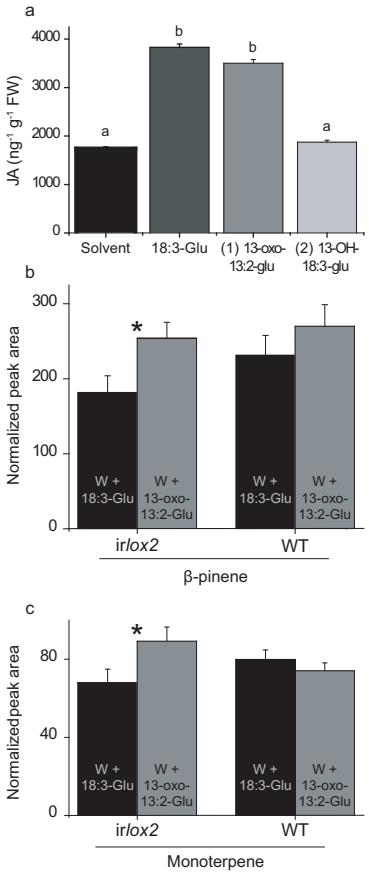

Figure 6 Analysis of JA and terpenoid volatiles in WT and ir*lox2* plants after elicitation. **(a)** WT plants were wounded and of the wounds immediately treated with either solvent alone, 18:3-Glu (0.17 nmoles), 13-oxo-18:3-Glu or 13-OH-18:3-Glu (see text for a description of the amounts used). Samples were taken after 60 min and JA quantified by LC-MS/MS (n = 3, bars denote ± SE). Different letters denote significant differences (univariate ANOVA $F_{3,3} = 17.9$, $P < 0.001$ followed by a Scheffé *post-hoc* test, $P < 0.05$). **(b,c)** ir*lox2* and WT plants were wounded and treated with either 18:3-Glu (0.17 nmoles) or 13-oxo-13:2-Glu (see text for a description of the amounts used), the emitted volatiles were trapped for 8 h after 24 h of the treatment and analyzed by GCxGC-ToF. Injection of a β-pinene standard confirmed the structure of the first compound, while the second monoterpene remained unidentified. The MS spectra of the two compounds are shown in Additional file 3. Asterisks indicate significant difference ($P < 0.05$, Student's *t*-test).

Discussion

In this study, we demonstrated that one of the major elicitors present in the OS of *M. sexta* larvae, *N*-linolenoylglutamate (18:3-Glu), was rapidly oxidized by a LOX-dependent reaction upon contact with wounded leaf tissue; *N. attenuata* plants silenced in the expression of *NaLOX2* and *NaLOX3* (*irlox2* and *3*, respectively) were affected in their capacity to metabolize 18:3-Glu into its oxidized forms. This process occurred when either synthetic 18:3-Glu or OS were applied onto wounded leaves. Metabolism of 18:3-Glu was however slower when OS was applied, suggesting that the *M. sexta* OS may contain either inhibitors of the LOX-dependent reaction or that the FACs are less accessible to LOXs (e.g., by interacting with other OS components). The formation of the LOX product 13-OOH-18:3-Glu occurred within seconds and based on the metabolism of radioactive ^{14}C-18:3-Glu, we estimated that after 2 min of contact with wounded leaf tissue, 55 to 60% of the applied 18:3-Glu was metabolized by LOX activity. These results suggested that LOXs can rapidly utilize 18:3-Glu as a substrate and catalyze its 13-hydroperoxidation. ir*lox2* and ir*lox3* plants showed similar rates of 18:3-Glu metabolism (Fig. 5b), and due to the fact that ir*lox3* plants have also significantly reduced levels of NaLOX2 transcripts whereas ir*lox2* plants have WT levels of NaLOX3 transcripts [29], we conclude that most likely NaLOX2 is the major LOX isoform involved in 18:3-Glu metabolism. Without mechanical damage, 18:3-Glu was not metabolized in contact with leaf surfaces, suggesting that mechanical disruption of leaf cells releases LOX enzymes into the extracellular space where they come into rapid contact with 18:3-Glu. Consistently, heat treatment of the wounded leaves strongly reduced the metabolism of 18:3-Glu and prevented the accumulation of its LOX-dependent derivatives. Non-enzymatic mechanisms resulting in derivatives different from those produced by LOX activity (Supplemental file 1) were most likely responsible for the partial metabolism of 18:3-Glu after heat inactivation of the leaves (Fig. 5a).

In a previous study, beet armyworm caterpillars fed with radiolabeled plant material were allowed to feed on unlabeled maize plants, and the results showed that this FAC is transferred from the caterpillar's OS into the feeding site of the leaf [21]. In the same study, however, there is no indication of a plant-mediated conversion of volicitin, as no additional radioactive fractions could be recovered from leaf tissue after caterpillar feeding. These results may indicate that the presence of the hydroxyl group at the C_{17} position of the fatty-acid moiety of volicitin inhibits the lipoxygenase-mediated conversion of this insect elicitor.

Hydroperoxy fatty acids are substrates for a diverse set of enzymatic and non-enzymatic reactions in plant tissues [31]. Among the reactions involving 13-OOH-18:3 are the reduction of the hydroperoxy group into a hydroxyl group to form 13-OH-18:3 and the cleavage of the C_{13}-C_{14} bond to generate 13-oxo-C_{13} derivatives [25,26]. Consistent with these reactions we observed the formation of 13-oxo-13:2-Glu and 13-OH-18:3-Glu on the leaf surface of wounded *N. attenuata* plants. Similar to 13-OOH-18:3-Glu, the formation of these molecules was detected within seconds upon contact with wounded leaf tissue and based on the metabolism of radioactive ^{14}C-18:3-Glu, we estimated that together they accounted for approximately 20 to 25% of the initial amounts of 18:3-Glu applied (after 2 min of contact with wounded leaf tissue). Whether these two 13-OOH-18:3-Glu derivatives are produced via enzymatic or non-enzymatic mechanisms on the leaf surface is at present unknown. Formation of 13-oxo-13:2-Glu from 13-OOH-18:3-Glu requires the cleavage of the α-C_{13}-C_{14} bond and in soybean seeds an enzymatic activity that cleaved 13-OOH-18:3 into 13-oxo-trideca-9,11-tridecanoic acid and two isomeric pentenols has been described [25]. In animal cells, this reaction (reductive β-scission) has been proposed to be mediated by cytochrome P-450 enzymes [32]. Formation of 13-oxo-13:2 from 13-OOH-18:3 can also occur non-enzymatically by thermal decomposition [25,26], however, analysis of 18:3-Glu metabolism in the presence of a radical scavenger (BHT) and a reducing agent (TMP) [27] showed that this conversion took place on the leaf surface (Additional file 2). Whether this conversion occurs enzymatically or not in wounded leaves remains unknown. In the case of 13-OH-18:3-Glu, the reduction of the hydroperoxy group into a hydroxyl group is the most plausible mechanism and as mentioned above, the mechanism involved remains to be elucidated.

Elicitation of *N. attenuata* leaves with purified 13-oxo-13:2-Glu was sufficient to enhance JA production to levels similar to those induced by 18:3-Glu (Fig. 6a), indicating that this oxidized form of 18:3-Glu is active as an elicitor and that its relative activity is similar to that of unmodified 18:3-Glu in terms of JA induction. In contrast to 13-oxo-13:2-Glu, 13-OH-18:3-Glu was inactive in mediating an enhanced JA production, suggesting that some modifications could be important for the rapid inactivation of FACs and therefore for the control of the FAC-mediated elicitation stimulus. The activity of 13-oxo-13:2-Glu was also evidenced by the differential induction of two emitted monoterpenes (β-pinene and a monoterpene of unidentified structure) in ir*lox2* plants (Fig. 6b,c). In WT plants this difference disappeared, most likely because of a high 18:3-Glu conversion to 13-oxo-13:2-Glu. All together, our results suggest a degree

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of specificity in the responses elicited by modified forms of 18:3-Glu.

Conclusions

The results presented showed that upon contact with wounded *N. attenuata* leaves, the FAC elicitor 18:3-Glu is rapidly metabolized by LOX activity to form additional active and inactive elicitors. In particular, 13-oxo-13:2-Glu was active as an elicitor of an enhanced JA biosynthesis and of the differential emission of two monoterpenes. Although speculative at this point, the results presented open the possibility that the metabolism of 18:3-Glu may play a role in the tuning of some plant responses to insects. Future investigations will be focus on the unraveling of these potential responses.

Methods

Plant growth and treatments

Seeds of the 22th generation of an inbred line of *Nicotiana attenuata* plants were used as the wild-type (WT) genotype in all experiments. Plants were grown at 26-28°C under 16 h of light. In all experiments, slightly elongated *N. attenuata* plants were used. For 18:3-Glu elicitation experiments, puncture wounds were generated using a fabric pattern wheel, wounds were immediately supplied with 10 μL of a solution containing 0.17 nmoles of synthetic *N*-linolenoyl-glutamic acid (18:3-Glu; dissolved in 0.02% (v/v) Tween-20/water). For elicitation with purified oxidized forms of 18:3-Glu, amounts corresponding to ion intensities (as analyzed by LC-MS/MS; see below) similar to those detected after 2 min of 18:3-Glu metabolism on wounded leaves were used. Similar to 18:3-Glu, these modified forms were dissolved in 0.02% (v/v) Tween-20/water. The total treated area was quickly excised and used immediately for extraction and subsequent analysis. The oral secretion (OS) treatment was performed similarly but the wounds were supplemented with freshly harvested OS from *M. sexta* larvae (3rd to 5th instar) reared on *N. attenuata* plants. The amounts of 18:3-Glu in the OS were quantified by LC-MS/MS (see conditions below). For the steam treatment, three *N. attenuata* leaves were exposed to steam for 2 min and treated as above.

Synthesis of ^{14}C-labeled 18:3-Glu and turnover analysis

Ten μCi of [1-^{14}C]-9,12,15-linolenic acid (51.7 mCi/mmol, Perkin-Elmer, Rodgau, Germany) were dissolved in 2 mL of dry tetrahydrofurane containing 27.5 μL (0.198 mmol) of triethylamine. While stirring, 19 μL (0.197 mmol) of ethylchloroformate were added at 0°C (ice-water). After 3 min, 20 mg Glu dissolved in 1.4 mL 0.3 N NaOH were added. After 5 min, the ice bath was removed and the mixture stirred for 30 min at room temperature. The reaction was adjusted to pH 3-4 with 5 N HCl and extracted 3 times with 3 mL of dichloromethane. The combined organic phases were dried with Na_2SO_4 and evaporated to dryness under N_2. For purification, a column of 3 g of silica 60 gel was preconditioned with 100/1 (v/v) chloroform/acetic acid. The sample reconstituted in 1 mL 100/1 (v/v) chloroform/acetic acid was loaded onto the column. Washes were: 5 mL of 100/1 (v/v) chloroform/acetic acid two times, 5 mL of 14/6/1 (v/v/v) chloroform/ethylacetate/acetic acid two times collecting the flow-through in between. Purity of the fractions was checked by TLC using 14/6/1 (v/v/v) chloroform/ethylacetate/acetic acid as the solvent system.

^{14}C-18:3-Glu (0.1 μCi; 1.9 nmoles) were applied onto leaf wounds (area of 4 cm^2) and tissue collected at 0, 1 and 2 min. Leaf material was extracted two times with 2 ml of ethylacetate and radioactivity was quantified by liquid scintillation (WinSpectral model; Hewlett-Packard, Boeblingen, Germany). Metabolites were separated by TLC on silica gel 60 plates (Merck, Darmstadt, Germany) using 14/6/1 (v/v/v) chloroform/ethylacetate/acetic acid as the solvent system. After drying, TLC plates were exposed to ^{14}C-sensitive screens and the screens scanned with an FLA-3000 densitometric scanner (Fujifilm, Düsseldorf, Germany). Commercial α-linolenic acid (18:3; Sigma, Taufkirchen, Germany) was co-run as a standard. For radio-HPLC analysis, radioactive extracts were run on an HPLC system (Agilent HPLC 1100 Series, Palo Alto, CA), using a gradient of solvent A (0.05% (v/v) formic acid/water) and solvent B (0.05% (v/v) formic acid/acetonitrile) starting with a linear gradient of 20% to 70% (v/v) solvent B for 20 min, 70% (v/v) solvent B for 5 min, and 20% (v/v) solvent B for 5 min. The extract was separated with an RP Sphinx column (C_{18} and propylphenyl stationary phase, 15%C, 250 × 4.6 mm, 5 μm particle diameter, Macherey-Nagel, Düren, Germany) with a flow of 1 mL min^{-1}. For radiodetection, a flow scintillation analyzer 500 TR (Packard), using Ultima-Flo AP (Perkin Elmer, Jügesheim, Germany) scintillation liquid was used.

Purification and identification of 18:3-Glu derivatives

Ten mg of synthetic 18:3-Glu were dissolved in 0.5 mL water containing 0.02% (v/v) Tween-20 and applied to 20 wounded fully expanded leaves (500 μg leaf^{-1} or 1.2 μmoles leaf^{-1}). After 5 min, the treated leaves were cut and dipped for 30 seconds in 2:1 (v/v) chloroform/methanol. The solvent was evaporated under a gentle stream of nitrogen preventing heating, reconstituted in 70% (v/v) methanol/water and fractionated using the retention times from the radio-HPLC, with the same system connected to a fraction collector. After fractionation, samples were concentrated and injected in an ESI-ToF (MicroToF, Bruker Daltonics, Bremen, Germany)

system connected to an HPLC system (Agilent HPLC 1100 Series) equipped with a Phenomenex Gemini NX 3 μm column (150 × 2 mm) using the same solvents (A and B) as above. The gradient was first isocratic at 5% (v/v) solvent B for 2 min, and then a linear gradient to 80% (v/v) solvent B for 28 min, 80% (v/v) solvent B for 6 min, and 5% (v/v) solvent B for 9 min at a flow rate of 0.2 mL min^{-1} was used. Compounds were analyzed in the negative ion mode. Instrument settings were as follows: capillary voltage 4500 V, capillary exit 130 V, drying gas temperature 200°C, drying gas flow of 8 L min^{-1} and a ToF acceleration voltage of 2100 V. Ions were detected from *m/z* 100 to 1400. Using a syringe pump, samples were directly injected into an ESI-MS/MS (Varian 1200 Triple-Quadrupole-LC-MS system; Varian, Palo Alto, CA) system to confirm their identification as an 18:3-Glu derivative. 18:3-Glu derivatives were further purified by separation on preparative TLC silica gel 60 plates (Merck) and TLC fractions were eluted sequentially with 5 mL of dichloromethane, chloroform and ethylacetate. Fractions were concentrated under nitrogen and reconstituted in 70/30 (v/v) methanol/water for subsequent LC-ESI-ToF and LC-ESI-MS/MS analysis. For final structural elucidation, samples were injected on an LC-ESI-XL-Orbitrap (Thermo, Steingrund, Germany) using the linear ion trap for fragmentation. Conditions were: source voltage: 4050V, capillary voltage: -40V and a sheath gas flow rate of 25 L min^{-1}.

Extraction and analysis of JA and FACs

For analysis of JA, ~0.2 g of frozen leaf material was added to 2 mL SafeLock® (Eppendorf) tubes containing two steel beads, and homogenized in a Genogrinder® Model2000 (Munich, Germany) at 500 strokes min^{-1}. 1 mL ethylacetate spiked with 100 ng of [9,10-^{2}H]-dihydro-JA was added as an internal standard (IS), the samples were vortexed for 5 min and centrifuged under refrigeration (4°C) for 15 min at 13,200 × *g*. The upper organic phase was transferred to a fresh tube and the leaf material was re-extracted with 0.5 ml ethylacetate. The organic phases were pooled and evaporated to dryness. The dry residue was reconstituted in 0.4 mL of 70/30 (v/v) methanol/water for analysis by LC-ESI-MS/MS using previously described conditions [30].

FACs were extracted from leaves with 1 ml of ice-cold chloroform and chloroform/methanol 4/1 (v/v) with or without the addition of 1% (w/v) butylated hydroxytoluene (BHT; Sigma) and 10 mg/ml trimethyl phosphite (TMP; Sigma)[27] using the same grinding conditions as for JA extraction. The samples were spiked with 100 ng of [9,10-^{2}H]-dihydro-JA as an internal standard (IS) for normalization. The solvent was evaporated under a gentle stream of nitrogen keeping the samples on ice. Precautions were taken not to completely dry the samples and the residue was reconstituted in 0.4 mL of 70/30 (v/v) methanol/water for analysis with an LC-ESI-MS/MS system (Varian 1200 Triple-Quadrupole-LC-MS system). 10 μL of the sample was injected onto a ProntoSIL® column (C18, 5 μm, 50 × 2 mm, Bischoff, Leonberg, Germany) connected to a precolumn (C18, 4 × 2 mm, Phenomenex). As mobile phases 0.05%/1% (v/v/v) formic acid/acetonitrile/water (solvent A) and 0.05% (v/v) formic acid/acetonitrile (solvent B) were used, starting with 15% (v/v) solvent B for 1.5 min (pre-run), a linear gradient to 98% (v/v) solvent B for 3 min, 98% (v/v) solvent B for 8 min and 15% (v/v) solvent B for 2.5 min. Flow rates were: 0.4 mL min^{-1} for 1 min and 0.2 mL min^{-1} from 1 to 12 min, and 0.4 mL min^{-1} till the end of the run (15 min). Compounds were detected in the ESI negative mode and multiple reaction monitoring (MRM; see Additional file 4 for details on ion transitions and conditions used for analysis).

Volatile collection and GCxGC-ToF analysis

ir*lox2* and WT plants were induced with either wounding plus 18:3-Glu or wounding plus 13-oxo-13:2-Glu and the volatiles emitted by the induced leaves were trapped as described previously [17]. Briefly, a single leaf was enclosed in a plastic volatile collection chamber and volatiles were trapped on 20 mg of Super-Q absorbent (ARS, Philadelphia, PA) secured with glass wool in small glass cylinders. Ambient air filtered through activated charcoal was pulled at 200 to 300 ml min^{-1} into each collection chamber with a vacuum pump. Volatile trapping was performed after 24 h of the treatment for a period of 8 h. Traps were spiked with 400 ng tetraline as IS and eluted with 250 μL of dichloromethane. Eluted volatiles were injected into a GCxGC-ToF system (Leco, Germany) and the samples run using the same instrument parameters as previously described [17]. For analysis, a reference sample made by mixing all the different samples was injected and a reference library was created by software assisted peak finding. Non-relevant peaks (e.g. plasticizers) were manually removed, and all individual samples were processed against this reference. After manual correction of the peak integrated areas, data was normalized by the IS (tetraline) and the total ion current (TIC). Significant peaks were identified using an unpaired student's *t*-test.

Data analysis

All experiments were performed with at least three individual plants (biological replicates). Statistics were calculated using SPSS v. 17.0, data was *log*-transformed when the data was not homoscedastic.

Additional material

Additional file 1: Metabolism of ^{14}C labeled 18:3-Glu in wounded *N. attenuata* leaves.

Additional file 2: Analysis of 13-oxo-13:2-Glu biogenesis on the leaf surface.

Additional file 3: Mass spectra of two monoterpenes detected by GC-MS.

Additional file 4: List of ion transitions used for analysis of compounds by LC-MS/MS.

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Authors' contributions
AVD and GB carried out the experiments, analyzed the data and drafted the manuscript. AAB carried out experiments and analyzed the data. MK analyzed the data. ITB participated in the design and coordination of the study and helped to draft the manuscript. GB conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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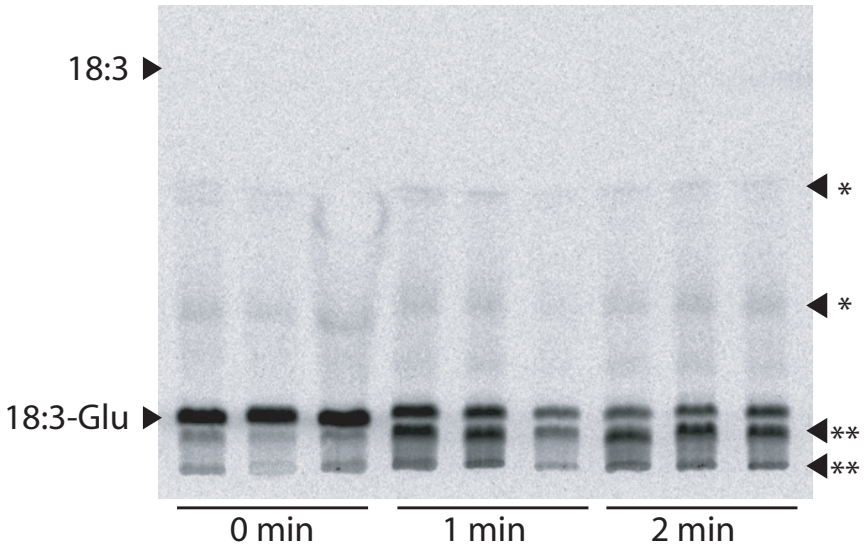
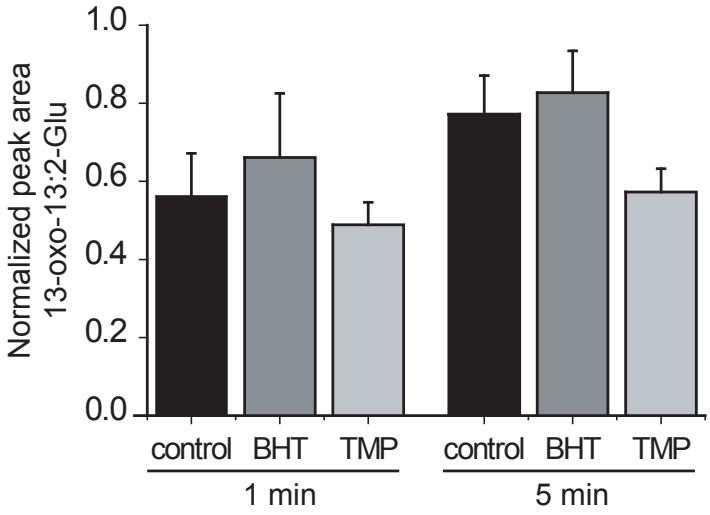
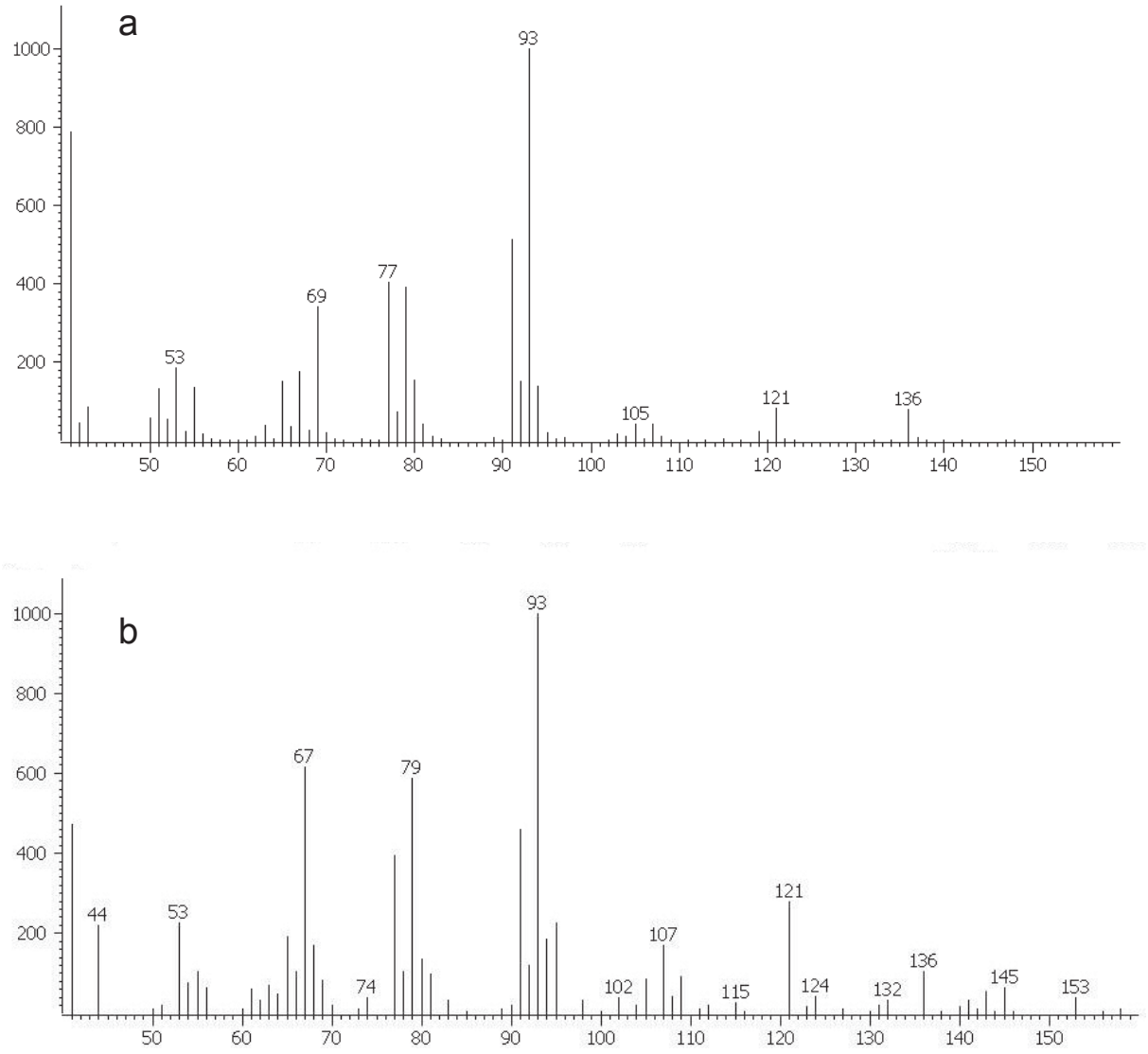


Additional file 1. Metabolism of ^{14}C labeled 18:3-Glu on wounded *N. attenuata* **leaves** Leaves of rosette stage *N. attenuata* plants were wounded with a pattern wheel and 0.1µCi of [1- ^{14}C]18:3-Glu were immediately applied onto leaf wounds. The damaged tissue was harvested immediately (T0) and 1 and 2 min after the treatment and extracted. Radiolabeled metabolites were separated by TLC and plates were exposed for 24h to ^{14}C-sensitive screens. Major (**) and monor (*) [1-^{14}C]18:3-Glu derivatives were marked on the right, α-linolenic acid (18:3) and 18:3-Glu were co-run and their position of migration indicated on the left.



Additional file 2. Analysis of 13-oxo-13:2 biogenesis on the leaf surface. WT plants were wounded with a pattern wheel and 0.17 nmoles of 18:3-Glu were applied onto the wounds. After 1 and 5 min, leaf tissue was extracted without (control) or with the addition of butylated hydroxytoluene (BHT) or trimethyl phosphite (TMP) to the solvent. After extraction, samples were analyzed by LC-MS/MS (n=3, bars denote ± SE). Univariate *ANOVA* (1 Min $F_{3,3}$=0.799, P=0.492; 5 Min $F_{3,3}$=2.166 P=0.196).



Additional file 3. Mass spectra of monoterpenes detected by GC-MS a. Mass spectrum of β-pinene. b. Unknown monoterpene. Average mass spectra were recorded after background substraction and compared against standards. β-pinene matched in both the mass spectrum and both retention times.

Name of analyte	Molecular ion [M-1]	Fragment ion	Capillary CID	Collision energy
JA	209	59	-35V	12V
$^{2}H_2$-dihydro-JA	213	59	-35V	12V
18:3-Glu	406	128	-35V	21.5V
13-OOH-18:3-Glu	438	352	-20V	19V
13-OH-18:3-Glu	422	293	-35V	18V
13-oxo-13:2-Glu	352	128	-35V	18V

Additional file 4. List of ion transitions used for analysis of compounds by LC-MS/MS.

Chapter 3

Herbivore Associated Molecular Patterns: FAC Signaling and Metabolism

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Herbivore Associated Molecular Patterns: FAC signaling and metabolism

Abstract

The recognition of insect and pathogen attack requires the plant's ability to perceive chemical cues generated by the attacker. In contrast to the recognition of microbe-associated molecular patterns (MAMPs), little is known about the molecular recognition of herbivore-associated molecular patterns (HAMPs) and the signaling mechanisms operating in plants after their perception. HAMP perception and triggered responses depend strongly on the natural history of both plants and insects and it is therefore expected that a large number of the responses induced by different HAMPs are specific to the species involved in the interaction. The interaction between *Nicotiana attenuata* and the specialist Lepidoptera *Manduca sexta* presents a relevant system to understand the evolution of HAMP-mediated responses in plants.

Glossary:

HAMPs (Herbivore-associated molecular patterns): Molecules derived from insect herbivores or from herbivore feeding activity that plants recognize to modulate defence or tolerance responses.

MAMPs (Microbe-associated molecular patterns): Molecules derived from microorganisms that plants recognize to modulate defence or association responses.

FACs (Fatty acid-amino acid conjugates): Molecules present in the oral secretions/saliva of lepidopteran larvae and other species.

Volicitin (N-(17-hydroxylinolenoyl)-L-Gln): The first FAC identified from the oral secretion of *Spodoptera exigua* larvae.

18:3-Glu (*N*-linolenoyl glutamic acid): One of the most abundant FACs in the oral secretions of *Manduca sexta* larvae.

N. attenuata (*Nicotiana attenuata*): An annual tobacco plant native to the deserts of the Southwestern USA.

M. sexta (*Manduca sexta*): A moth of the family *Sphingidae* commonly known as the tobacco hornworm.

NaWIPK (*N. attenuata* Wound-induced protein kinase): A mitogen-activated protein kinase (MAPK) first described as a wound induced protein kinase in *N. tabacum*. It has been associated with responses to herbivory in *N. attenuata*.

NaSIPK (*N. attenuata* Salicylic acid-induced protein kinase): A mitogen-activated protein kinase (MAPK) first described as a SA-induced protein kinase in *N. tabacum*. It has been associated with responses to herbivory in *N. attenuata*.

NaNPR1 (*N. attenuata* Non-expressor of PR-1): First identified in *A. thaliana* as a component of the SA signal transduction machinery, it was later associated to responses against herbivores in *N. attenuata*.

NaLOX2 (*N. attenuata* Lipoxygenase-2): A 13-LOX involved in the formation of 13S-hydroperoxide derivatives of polyunsaturated fatty acids for green leaf volatile production.

NaLOX3 (*N. attenuata* Lipoxygenase-3): A 13-LOX involved in the formation of 13S-hydroperoxide derivatives of polyunsaturated fatty acids for JA biosynthesis.

NaGLA1 (*N. attenuata* Glycerolipase-A1): The first enzyme in the leaf JA biosynthesis pathway catalyzing the release of linolenic acid from chloroplast membrane glycerolipids.

NaAOS (*N. attenuata* Allene oxide synthase): The 3rd enzyme in the JA biosynthesis pathway that uses 13*S*-hydroperoxide derivatives of polyunsaturated fatty acids to form an unstable allene oxide.

NaAOC (*N. attenuata* Allene oxide cyclase): The 4th enzyme in the JA biosynthesis pathway that cyclases the allene oxide produced by AOS to generate (9*S*,13*S*)-12-oxo-phytodienoic acid (OPDA).

SuperSAGE (Super Serial analysis of gene expression): A technique used to quantify changes in gene expression that depends on the generation and identification of 26 bp sequences (Tags) originated from individual mRNAs.

HAMPs are specific elicitors of plant responses to insect folivory

A large number of plant species can distinguish insect folivory from mechanical damage by the perception of chemical cues (herbivore-associated molecular patterns: HAMPs; see Glossary) generated by the insect. However, in contrast to the recognition of microbe-associated molecular

patterns (MAMPs) (Jones and Dangl, 2006), relatively little is known about the molecular recognition of HAMPs by plants and the signaling mechanisms operating after their perception. In general, perception of HAMPs results in the activation of specific plant responses to defend against or tolerate attack from insect herbivores; these responses can range from changes in metabolism (including changes in volatile emissions (Turlings *et al*., 1990; Mattiacci *et al*., 1995; Allmann and Baldwin, 2010)) and gene expression to changes in the pattern of growth and development (Landolt *et al*., 1999; Reymond *et al*., 2000; Schwachtje and Baldwin, 2008; Kessler et al., 2010). In some cases, HAMPs can also counteract some of the defense response of plants (Musser et al., 2002; Diezel et al., 2009).

The characterized HAMPs that act during insect folivory are diverse in structure, ranging from enzymes (e.g., glucose oxidase, β-glucosidase) (Mattiacci et al., 1995; Eichenseer et al., 1999) to modified forms of lipids (e.g., fatty acid-amino acid conjugates (FACs) (Alborn et al., 1997), sulfur-containing fatty acids (cealiferins) (Alborn et al., 2007)) and from fragments of cell walls (e.g., pectines and oligogalacturonides) (Doares et al., 1995; Bergey et al., 1999) to peptides released from digested plant proteins (e.g., inceptins: proteolytic fragments of the chloroplastic ATP synthase γ-subunit) (Schmelz et al., 2006).

Importantly, most of these HAMPs are not general elicitors of responses against insect herbivores in all plant species but are usually restricted to particular plant-insect associations (Table 1). This selectivity most likely reflects the evolutionary history of both plants and their interacting insects and hence it is critical to understand the mechanisms of plant-HAMP interactions in the evolutionary context of the interaction. The inability of known HAMPs to function as general elicitors of plant responses to herbivores has led to some controversy and part of the scientific community remains skeptical about the importance of HAMPs as specific elicitors of plant responses. Additionally, based on the small amounts of oral secretions (OS) commonly deposited by caterpillars onto wounded plant tissue during feeding, a major role of HAMPs has been questioned (Peiffer and Felton, 2009). Finally, HAMPs have been defined as plant damage-derived molecules perceived by self-recognition mechanisms (Heil, 2009); a definition that may encompass HAMPs derived from cell wall fragments or peptides from plant digested proteins but that does not address elicitors that are synthesized by the insects (such as modified forms of lipids or insect enzymes) (Pare et al., 1998; Musser et al., 2002; Lait et al., 2003).

Part of the controversy originates from our lack of knowledge about the molecular components operating in plants to recognize HAMPs; how they are perceived and how their perception is

translated into downstream signaling events leading to the activation of specific physiological processes.

Table 1. The perception of HAMPs is specific to the species of interacting and insects.

HAMPs	Insect species[a]	Plant species[b]
Glucose Oxidase (GOX)	*Helicoverpa zea* (Musser *et al.*,, 2002) *Spodoptera. exigua* (Diezel *et al.*, 2009) *Helicoverpa armigera* (Hu *et al.*, 2008) Other Lepidoptera and Hymenoptera (Eichenseer *et al.*, 2010)	*Nicotiana tabacum* (Musser *et al.*, 2002) *Nicotiana attenuata* (coyote tabacco) (Diezel *et al.*, 2009) *Medicago truncatula* (Hu *et al.*, 2008) *Solanum lycopersicum* (tomato) (Eichenseer *et al.*, 2010)
β-glucosidase	*Pieris brassicae* (Mattiacci *et al.*, 1995)	*Phaseolus-lunatus* (lima beans) (Hopke *et al.*, 1994) *Zea-Mays* (maize) (Hopke *et al.*, 1994) *Brassica oleracea* (cabbage) (Mattiacci *et al.*, 1995)
N-acyl-amino acids (FACs)	*Spodoptera exigua* (Alborn *et al.*, 1997) *Manduca sexta* (Halitschke *et al.*, 2001) *Teleogryllus taiwanemma* (Yoshinaga *et al.*, 2007) *Drosophila melanogaster* (Yoshinaga *et al.*, 2007) Several Lepidoptera (Pohnert *et al.*, 1999; Yoshinaga *et al.*, 2007)	*Zea mays* (Alborn *et al.*, 1997, Schmeltz *et al.*, 2009) *Glycine max* (soybean) (Schmeltz *et al.*, 2009) *Solanum melongena* (eggplant) (Schmeltz *et al.*, 2009) *Nicotiana attenuata* (Halitschke *et al.*, 2001) *Solanum nigrum* [A. vanDoorn and G. Bonaventure, unpublished results]
Caeliferins	*Schistocerca americana* (Alborn *et al.*, 2007)	*Zea mays* (Alborn *et al.*, 2007) *Arabidopsis thaliana* (Schmeltz *et al.*, 2009)
Inceptin	Produced by degradation of a plant ATP synthase during folivory by *Spodoptera frugiperda* (Schmeltz *et al.*, 2006)	*Vigna unguiculata* (cowpea) (Schmeltz *et al.*, 2006) Some *Fabaceae* (Schmeltz *et al.*, 2009)
Oligouronides	Produced by degradation of plant cell walls during insect folivory	*Solanum lycopersicum* (Doares *et al.*, 1995)

[a] Represent some of the insect species in which the indicated HAMP has been detected in their oral secretions.
[b] Represent some of the plant species in which a differential response to the indicated HAMP has been reported.

Some studies have started to address this issue: one pioneering study has reported that volicitin binds to maize (*Zea mays*) plasmamembranes with properties that resemble those of a ligand-receptor interaction (Truitt *et al.,* 2004) and some recent studies have shown that early plant responses to insect herbivory include changes in ion fluxes across the plasmamembrane (Maffei *et al.,* 2004),

activation of protein kinases (Wu *et al.,* 2007; Kallenbach *et al.,* 2010) and generation of reactive oxygen species (Maffei *et al.,* 2006).

Importantly, the elicitors of M. sexta OS are active in minute amounts, comparable with real transfer rates during larval feeding (Schittko *et al.,* 2001). The feeding process of folivorous insects is associated to a temporally irregular consumption of small pieces of tissue (i.e. mechanical damage). Therefore –in those cases where the plant can recognize the herbivore or its derived HAMP(s)– herbivore feeding/HAMP elicitation must consist of a re-programming of the wound response.

FAC elicitation in Nicotiana attenuata as a model system to study HAMP-mediated elicitation

FACs are OS components of a large number of lepidopteran larvae (Yoshinaga *et al.,* 2010) and they are necessary and sufficient to elicit herbivory-specific responses in several plant species including maize (*Zea mays*) and wild-type tobacco (*Nicotiana attenuata*) (Alborn *et al.,* 1997; Halitschke *et al.,* 2001). Volicitin (N-(17-hydroxylinolenoyl)-L-Gln) was the first FAC identified from the OS of *Spodoptera exigua* caterpillars (Alborn *et al.,* 1997). Additional FACs have been identified in lepidopteran larvae (e.g., *Manduca* species) with major forms consisting of linoleic (18:2) and linolenic (18:3) acids conjugated to glutamate (Glu) or glutamine (Gln) (Yoshinaga *et al.,* 2010). In addition to lepidopteran larvae, FACs have also been detected in two cricket species (*Teleogryllus taiwanemma* and *T. emma*) and in the larvae of *Drosophila melanogaster* (Yoshinaga *et al.,* 2007), suggesting that these HAMPs are broadly taxonomically distributed.

The longstanding question of why lepidopteran larvae would produce these potent elicitors was addressed in a recent study demonstrating the essential role of FACs in nitrogen assimilation by the larvae (Yoshinaga *et al.,* 2008). Thus, plants perceive molecules that are essential for the caterpillar's growth and development, that "tag" the damage resulting from herbivore attack, and it would be difficult for the caterpillars to do without, in order to feed "stealthly".

Nicotiana attenuata is an annual tobacco plant native to the deserts of the Southwestern USA and in its natural habitat it is attacked by a plethora of insects from different feeding guilds and among them, the foliviorous larvae of the specialist *Manduca sexta* (*M. sexta*) is frequently responsible for a majority of leaf area lost to herbivores in natural populations. *N. attenuata* can identify *M. sexta* larval folivory through the recognition of the FACs in the caterpillar's OS; synthetic FACs can induce responses almost identical to those induced by *M. sexta* folivory (Halitschke *et al.,* 2001; Giri *et al.,* 2006; Gaquerel *et al.,* 2010; Gilardoni *et al.,* 2010). Thus, the study of FAC signaling and perception in *N. attenuata* represents an excellent system with which to tackle some of the open

questions in the field of HAMP perception/signaling: How are HAMPs perceived and how do they signal the activation of responses to ward off or tolerate herbivores?

Three recent studies have started to disentangle the effect of FAC elicitation to provide important information on: 1) how these elicitors are metabolized on wounded leaf tissue to generate new active signals that can be perceived by the plant perhaps to tailor responses (Vandoorn *et al.,* 2010); 2) how they control the enhanced activation of jasmonic acid (JA) biosynthesis (Kallenbach *et al.,* 2010); 3) how they affect the expression of early regulated genes encoding potential signal transduction components (Gilardoni *et al.,* 2010). We briefly described these discoveries and put forward new hypothesis that arise from them.

FACs are rapidly metabolized on wounded leaves to generate active and inactive signal molecules

When the metabolism of *N*-linolenoyl glutamic acid (18:3-Glu) was analyzed on *N. attenuata* wounded leaf surfaces, it was found that a large fraction (ca. 50-70%) of this FAC in applied *M. sexta* OS or of applied synthetic 18:3-Glu was metabolized within a few seconds (Vandoorn *et al.,* 2010). The metabolism of 18:3-Glu did not produce free α-linolenic acid (18:3) and glutamate as expected if the amide bound of the molecule was hydrolyzed but instead this FAC was converted into an array of more and less polar derivatives by a heat-labile process. The three major modified forms of 18:3-Glu were identified as 13-hydroxy-18:3-Glu, 13-hydroperoxy-18:3-Glu and 13-oxo-13:2-Glu; typical oxidation products of reactions catalyzed by 13-lipoxygenases. Indeed, the oxidation of 18:3-Glu on wounded leaves was largely dependent on lipoxygenase-2 (NaLOX2); a plastid-localized enzyme feeding the C_6 volatile biosynthesis pathway in *N. attenuata* leaves (Allmann *et al.,* 2010). Upon tissue disruption, NaLOX2 likely comes into contact with the FACs deposited on the wound surface. The activity of this LOX towards 18:3 conjugates is consistent with previously described LOX activity towards membrane lipids and 18:2-ethanolamines (Feussner and Wasternack, 1998; Shrestha *et al.,* 2002).

Similar to the enhanced activation of JA biosynthesis by intact 18:3-Glu, the 13-oxo-13:2-Glu derivative also enhanced the accumulation of this phytohormone, indicating that 13-oxo-13:2-Glu is perceived as an elicitor by wounded leaves. In contrast, 13-hydroxy-18:3-Glu was inactive in the eliciton of JA biosynthesis, suggesting that the hydroxylation of 18:3-Glu may function as an inactivation mechanism of the elicitor (in order to prevent a continuous stimulation). In addition to triggering the enhanced accumulation of JA, 13-oxo-13:2-Glu also induced the differential emission of two monoterpene volatiles (β-pinene and an unidentified monoterpene) in plants deficient in NaLOX2 activity.

These results led to the hypothesis that the metabolism of 18:3-Glu tunes some defense responses to insect attack. Additionally, its metabolism may occur to rapidly inactivate a fraction of the 18:3-Glu and therefore to control the elicitation process. In addition to the three derivatives described above, at least ten other different forms were detected by analysis of radiolabeled 18:3-Glu (VanDoorn et al., 2010), suggesting the possibility that different modified forms are active in the differential stimulation of distinct *N. attenuata* responses (Fig. 1). Whether these responses depend on different receptors is an intriguing question.

Box 1: Highlight of new perspectives

The identification of the mechanisms involved in the FAC-mediated enhanced production of JA and dependent on NaSIPK, NaWIPK and NaNPR1 in *N. attenuata* will provide critical insights into the mechanisms used by the cells to convey the primary stress signal (wounding plus FAC perception) into the activation of JA biosynthesis. These mechanisms could include post-translational modifications of enzymes to promote their interactions with membranes or other partners in the pathway. We hypothesize that perception of FACs by wounded leaves differentially activates the association of NaGLA1 with NaLOX3 or the activity of the complex to induce an enhanced supply of substrates for JA biosynthesis.

The identification of additional components affecting early enzymatic steps of the JA biosynthesis pathway in other plant species (Ludwig *et al.*, 2005; Takabatake *et al.*, 2006; Tajahashi *et al.*, 2007; Schweighofer *et al.*, 2007) will provide important insights into the network of components that regulate the activation of JA biosynthesis and whether or not these components are involved in HAMP/FAC-mediated signal transduction pathways.

The rapid metabolism of 18:3-Glu on wounded *N. attenuata* leaves points to a possible involvement of FAC modified forms in to the differential elicitation of defense responses. The identification of additional 18:3-Glu modified forms generated in wounded *N. attenuata* leaves plus the analysis of the metabolism of additional FACs (e.g., 18:2-Gln) will be important to determine whether these derivatives have a specific function in tuning plant responses. These studies can be extended to the metabolism of other HAMPs/FACs in different plant species and be used to investigate their implications for plant defense and/or tolerance responses to insects.

Gene expression profiling in *N. attenuata* leaves disclosed a number of genes which are only induced or repressed by FAC elicitation but which are unresponsive to mechanical damage. A significant percentage of these genes encode for putative regulatory components and may represent regulatory genes acting early in the induction of responses to *M. sexta* folivory. The elucidation of the role of these genes in *N. attenuata* may shed light into the mechanisms that plants use to decode FACs and thereby to induce specific defensive or tolerance responses. This goal can be achieved by the systematic study of the function of these genes in plants silenced in their expression.

Forward genetics approaches in plant species like Arabidopsis may prove instrumental in the identification of signal transduction components sensitive to specific HAMPs and regulating , for example, the differential accumulation of JA or ethylene in this plant species (Schmelz *et al.*, 2009).

FAC elicitation differentially activates early enzymatic steps of the JA biosynthesis pathway

Plastids are central orchestrators of some of the early responses to wounding and herbivory in plants; this organelle houses some of the most important enzymes involved in the biogenesis of signals that mediate responses against these stresses (e.g., the enzymes initiating JA biosynthesis) (Bonaventure and Baldwin, 2010). In unelicited leaves, JA is maintained at very low levels however upon stimulation its biosynthesis is induced within a few minutes (Grata *et al.,* 2007; Kallenbach *et al.,* 2010). This rapid induction results from the activation of constitutively expressed biosynthetic enzymes. Importantly, the perception of FACs by wounded *N. attenuata* leaves amplifies the wound-induced accumulation of JA and its intermediates by 2 to 3 times (Kallenbach *et al.,* 2010).

In a recent study, (Kallenbach *et al.,* 2010) provided the first evidence for the participation of two mitogen activated protein kinases (NaSIPK and NaWIPK) and the NaNPR1 regulator in the activation of early enzymatic steps of the JA biosynthesis pathway after FAC elicitation in *N. attenuata* leaves. Analysis of the early changes in the pools of JA precursors showed that the enhanced accumulation of the NaLOX3 product 13*S*-OOH-18:3 after FAC elicitation occurred within 1 min and that it was independent of changes in 13-LOX activity. These results suggested that the supply of free α-linolenic acid (18:3) rather than LOX activity was regulated by FAC elicitation and responsible for the FAC-elicited JA burst. Moreover, accumulation of 13*S*-OOH-18:3 depended on the expression of NaSIPK and NaNPR1 and it was again independent of reductions in 13-LOX activity in ir-*sipk* and ir-*npr1* plants (*N. attenuata* plants silenced in the expression of either Na*SIPK* and Na*NPR1* genes). Consistent with the participation of a lipase in the supply of 18:3 to the pathway, a plastidial glycerolipase (Na*GLA1*) was identified and shown to be essential for de novo JA biosynthesis in *N. attenuata* leaves after both wounding and FAC elicitation. We hypothesize that NaGLA1 and/or NaLOX3 are likely targets of the wound- and FAC-mediated mechanisms for JA activation in *N. attenuata* leaves and that these mechanisms depend on the differential activation of NaSIPK and NaNPR1. These mechanisms may operate in a manner similar to the mechanism activating leukotriene biosynthesis in mammalian cells (Funk, 2001), for example, by inducing the interaction between the lipase and the lipoxygenase (NaGLA1 and NaLOX3 in this case) (Fig. 1).

In contrast to ir*sipk* and ir*npr1* plants, the curtailed accumulation of JA in ir*wipk* plants (*N. attenuata* plants silenced in the expression of the Na*WIPK* gene) could be attributed to a deficiency in the accumulation of 12-oxo-phytodienoic acid (OPDA); ir*wipk* plants accumulated WT levels of 13*S*-OOH-18:3 however their rates of OPDA accumulation were several fold reduced. These results are consistent with a NaWIPK-mediated control of allene oxide synthase (NaAOS) and/or allene oxide cyclase (NaAOC) activity. Thus, such a scenario would have FAC elicitation differentially activating NaWIPK, which in turn would tranduce the signal further into the plastid to control the activity of NaAOS and/or NaAOC (Fig. 1).

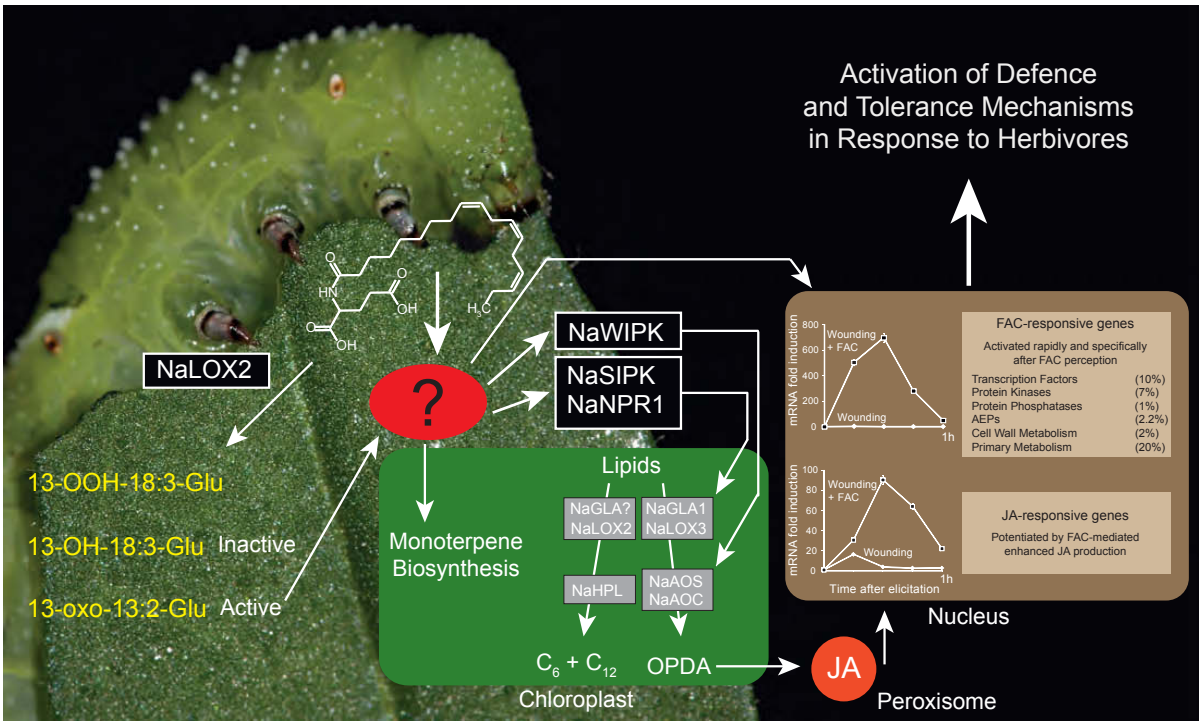

Figure 1 New insights into FAC signaling in *N. attenuata* leaves. Feeding by *M. sexta* larvae on *N. attenuata* leaves deposits FACs on the wound surface which are perceived by still unknown cellular components (red circle with question mark). One of the major FACs –18:3-Glu– in *M. sexta* OS is rapidly metabolized at the wound surface to generate oxygenated and other derivatives (VanDoorn *et al.*, 2010). NaLOX2, a chloroplast localized enzyme, comes into contact with 18:3-Glu upon tissue disruption and oxygenates 18:3-Glu to form active (13-oxo-18:3-Glu) and inactive (13-OH-18:3-Glu) elicitors of some defense responses (VanDoorn *et al.*, 2010). Perception of FACs and/or its modified forms triggers specific signaling events such as the differential activation of the MAPKs NaSIPK and NaWIPK together with NaNPR1. Activation of NaSIPK and NaNPR1 induces the release of 18:3 from membrane glycerolipids via the activation of NaGLA1 and the rapid conversion of this fatty acid into 13S-OOH-18:3 by NaLOX3 (Kallenbach *et al.*, 2010). This hydroperoxide is rapidly used for the synthesis of OPDA by NaAOS and NaAOC, a conversion stimulated by activation of NaWIPK (Kallenbach *et al.*, 2010). Perception of FACs also induces the specific activation of gene expression with a group of genes only activated when wounds come in contact with FACs but not in their absence (i.e. wounding alone). A substantial fraction of these genes correspond to putative regulatory components such as transcription factors and protein kinases (Gilardoni *et al.*, 2010) and may correspond to regulators of early responses against *M. sexta* caterpillars. NaSIPK and NaWIPK also contribute to the induction of some of these FAC responsive genes (Gilardoni, Baldwin and Bonaventure; unpublished results). A second group of genes is induced by mechanical damage and the induction heightened by FACs, most likely via the enhanced JA production after this stimulus. Abbreviations: OPDA: (9S,13S)-12-oxo-phytodienoic acid; JA: jasmonic acid; MT: monoterpenes; HPL: hydroperoxide lyase; AEPs: Avr-9 rapidly elicited proteins; C_6 and C_{12}: 6 and 12 carbon derivatives of the HPL pathway.

FACs induce rapid and specific changes in gene expression

Gene expression profiling using the SuperSAGE technique combined with next generation sequencing was used to quantify the early transcriptional changes specifically elicited by one of the major FACs in *M. sexta* OS, *N*-linolenoyl-glutamic acid (18:3-Glu) (Gilardoni *et al.*, 2010). The main objective of the study was to identify factors with potential regulatory functions during the *M.*

sexta-N. attenuata interaction; the analysis targeted mRNAs encoding regulatory components: rare transcripts with very rapid FAC-elicited kinetics. From 12,744 unique transcripts identified, 430 and 117 were differentially up- or down-regulated 30 min after 18:3-Glu elicitation compared to wounding. According to the expectations, a large percentage (25%) of these transcripts encoded putative regulatory components, including 30 transcriptional regulators and 22 protein kinases (PK). Interestingly, the expression of a large number of these transcripts was not altered by mechanical damage alone, indicating that FAC perception was essential for their regulation (Fig. 1). Among the transcriptional regulators were several WRKYs and AP2/ERF transcription factors (TFs), suggesting that these families of TFs orchestrate a large fraction of the early responses induced by FAC elicitation in *N. attenuata* plants (Skibbe *et al.,* 2008). Several of the transcripts encoding PKs belonged to the cell wall-associated protein kinase (WAK) family, proteins that may mediate changes in the cell wall structure in response to insect attack. Interestingly, several genes encoding Avr9-Cf9 rapidly elicited proteins (AEP) (Rowland *et al.,* 2005) were differentially induced by 18:3-Glu elicitation. The Avr9-Cf9 elicitor-receptor system is involved in the race-specific resistance of tomato (*S. lycopersicum*) against *Cladosporium fulvum* (Blatt *et al.,* 1999). Some of these transcripts may encode regulatory hubs in the network of the transduction pathways integrating signals for diverse elicitors (e.g., MAMPs, HAMPs) (Rowland *et al.,* 2005). This is consistent with the Cf9 receptor-mediated activation of WIPK and SIPK upon Avr9 binding in tomato (*Solanum lycopersicum*) (Romeis *et al.,* 1999), two MAPKs also involved in the wound and FAC elicitation responses in tobacco (Seo *et al.,* 1999; Seo *et al.,* 2007; Wu *et al.,* 2007).

Conclusions

In contrast to the fast advancing field of microbial elicitors, the molecular components used by plant cells to perceive HAMPs and to transduce and activate specific responses against folivorous insects remain largely unknown. Among the first identified elicitors of plant defense responses against pathogens (Ayers *et al.,* 1976; Nothnagel *et al.,* 1983; Roby *et al.,* 1985) and insects (Bishop *et al.,* 1981; Bishop and Ryan, 1987; Farmer *et al.,* 1991; Doares *et al.,* 1995) were fragments of plant cell walls that, in theory, could be interpreted as components of a self-recognition mechanism (Heil, 2009). Recent studies have however demonstrated that in the case of pathogens, the most prevalent MAMPs are molecules synthesized by the microorganisms and therefore detected by plants as foreign molecules (Nimchuk *et al.,* 2003; Jones and Dangl, 2006). Likewise, most of the HAMPs described thus far (Table 1) are synthesized by the insects and therefore their perception must rely on their detection as foreign rather than self molecules (Heil, 2009). One crucial question to advance the

field of HAMP perception and signaling is how we can identify the molecular components involved in these processes. A systematic RNAi-based approach to silence the expression of candidate regulatory genes in transgenic plants may be the fastest way forward in those cases where forward genetic approaches are difficult. For example, the systematic analysis of candidate *N. attenuata* regulatory factors rapidly induced by FACs may shed light into the mechanisms that plants use to decode these HAMPs (Box 1). Forward genetics approaches using plant species like Arabidopsis could also be used, for example, to identify components of the caeliferin-induced differential accumulation of JA or ethylene (Schmelz *et al.,* 2009).

The identification of the activation mechanisms regulating JA biosynthesis and which are enhanced by FAC perception and depend on NaSIPK, NaWIPK and NaNPR1 will provide critical insights into not only how JA biosynthesis is biochemically activated but also into the cellular components required for the FAC-mediated signal transduction of the primary stimulus into the plastids. The identification of additional signal transduction components in *N. attenuata* or other plant species affecting the activation of the JA biosynthesis pathway (Ludwig *et al.,* 2005; Takabatake *et al.,* 2006; Schweighofer *et al.,* 2007; Takahashi *et al.,* 2007) will provide further insights into the network of components that regulate the biosynthesis of this phytohormone after FAC/HAMP perception (Box 1).

Finally, the identification of additional 18:3-Glu modified forms generated in wounded *N. attenuata* leaves plus the analysis of the metabolism of additional FACs (e.g., 18:2-Gln) will reveal how these derivatives may tune plant responses. Studies of FAC metabolism in other responsive plant species like maize or *Solanum nigrum* (VanDoorn. Baldwin and Bonaventure, unpublished data) may also provide important information on the biological consequences of plant-modified FACs (Box 1). These studies can also be extended to the metabolism of other HAMPs by different plants and to their implications for plant defense and tolerance responses to insects.

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Chapter 4

Regulation of Jasmonate Metabolism and Activation of Systemic Defense Responses in *Solanum nigrum*: COI1 and JAR4 Play Overlapping, yet Distinct Roles

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Regulation of jasmonate metabolism and activation of systemic signaling in *Solanum nigrum*: COI1 and JAR4 play overlapping, yet distinct roles

Abstract

Jasmonates are ubiquitous messengers in land plants essential for the activation of defense responses. However, their signaling properties, accumulation and metabolism vary substantially among species. *Solanum nigrum* is a wild Solanaceous species developed as a model to study defense responses. *S. nigrum* plants transformed to silence the expression of key genes in jasmonate production (snLOX3), conjugation (snJAR4) and perception (snCOI1) were generated to analyze the function of these genes in jasmonate accumulation and metabolism (studied by a combination of LC-MS/MS and ^{13}C-isotope labeling methods) and in signaling (studied by the systemic elicitation of leucine aminopeptidase (LAP) activity). In contrast to the early single JA burst induced by wounding in WT plants, elicitation with insect oral secretions induced a later, second burst that was essential for the induction of systemic LAP activity, as demonstrated by ablation experiments. This induction was dependent on snLOX3 and snCOI1, but not on snJAR4. Additionally, the local accumulation of JA-glucose and JA-Ile was dependent on snCOI1, whereas the accumulation of hydroxylated jasmonates was dependent on both snCOI1 and snJAR4. The results demonstrate that snLOX3, snCOI1 and snJAR4 have overlapping yet distinct roles in jasmonate signaling, differentially controlling jasmonate metabolism and the production of a systemic signal.

Introduction

Jasmonic acid (JA) plays an essential role in mediating a plant's response to insect herbivores and JA accumulation and perception is critical for the elicitation of most defense-associated responses (Howe and Jander, 2008). JA biosynthesis starts in the chloroplast with the lipase-mediated release of 9,12,15-octadecatrienoic acid (18:3) and 7,10,13-hexadecatrienoic acid (16:3) (Ishiguro *et al.*, 2001; Ellinger *et al.*, 2010; Kallenbach *et al.*, 2010), which are subsequently dioxygenated by 13-lipoxygenases (LOXs) to form 13*S*-hydroperoxy-18:3 or -16:3. The plastidial enzymes allene oxide synthase (AOS) and allene oxide cyclase (AOC) convert the LOX product into (9*S*,13*S*)-12-oxo-phytodienoic acid (OPDA), which is then transported into the peroxisome where it is reduced by an OPDA reductase (OPR3) and β-oxidized to generate (3*R*,7*S*)-JA (Vick and Zimmermann, 1979; Bell *et al.*, 1995; Weber *et al.*, 1997; Stintzi and Browse, 2000; Park *et al.*, 2002; Delker *et al.*, 2006).

Conjugation of JA to isoleucine (Ile) to produce the active signal molecule jasmonyl-isoleucine (JA-Ile) has been shown to be important for the induction of many JA-mediated responses in several plant species. The biosynthesis of JA-Ile is catalyzed by JASMONIC ACID RESISTANT 1 (JAR1) in *Arabidopsis thaliana* (Staswick and Tiryaki, 2004) and by two isoforms, NaJAR4 and NaJAR6, in *Nicotiana attenuata* (Wang *et al.*, 2007). JA and JA-Ile accumulation is also herbivore specific in *N. attenuata*: when *Manduca sexta* oral secretions (OS) are applied to wounded leaves, both the JA and the JA-Ile bursts are two to three fold higher than after wounding alone (Kang *et al.*, 2006).

In addition to JA-Ile and other amino acid conjugates (Staswick and Tiryaki, 2004), JA can be converted into a diverse array of products, including isomerized, hydroxylated, glycosylated and sulfonated forms (Gidda *et al.*, 2003; Fonseca *et al.*, 2009). 12-hydroxy-jasmonyl (12-OH-JA) and 12-hydroxy-jasmonoyl-glucoside (12-*O*-glc-JA) were originally identified as tuber-inducing compounds in potato (Koda and Okazawa, 1988). However, recent studies have suggested that they may also play a role in the wound response (Miersch *et al.*, 2008; Seto *et al.*, 2009). Although no mechanism is known at this point, 12-OH-JA has been proposed as a negative regulator of JA biosynthesis (Miersch *et al.*, 2008), while a glycosyl transferase with 12-OH-JA glycosylation activity was found to be wound-inducible in *A. thaliana* (Seto *et al.*, 2009). The JA-Ile derivative, 12-OH-JA-Ile, was identified from *Solanum lycopersicum* and *A. thaliana* plants (Guranowski *et al.*, 2007), while another study identified 12-COOH-JA-Ile and showed that both oxygenated JA-Ile derivatives are wound-induced in *A. thaliana* plants (Glauser *et al.*, 2008). Importantly, although some enzymatic modifications of JA appear to occur in most plant species studied so far (e.g., JA-Ile formation), many are plant and tissue specific (Miersch *et al.*, 2008). How JA is converted into other forms and how this conversion is regulated is not very well understood. In *N. attenuata* plants NaCOI1 positively regulates JA-Ile turnover, resulting in a delayed accumulation of this hormone and in 20 to 30% higher JA-Ile levels in *coi1*-silenced plants compared to WT plants (Paschold *et al.*, 2008). However, for most modified forms of JA, the steps that regulate their biosynthesis or turnover remain largely unknown.

Advances in JA signaling have shown that JA-Ile is necessary for the interaction between the F-box protein CORONATINE INSENSITIVE 1 (COI1) (Xie *et al.*, 1998) and the JASMONATE ZIM DOMAIN (JAZ) proteins (Chini *et al.*, 2007; Thines *et al.*, 2007). JA-Ile perception by COI1 is essential in plant defense; *A. thaliana coi1* plants are more susceptible to necrotrophic pathogens and the insects *Pieris rapae* and *Myzus persicae* (Vijayan *et al.*, 1998; Mewis *et al., 2006*) while *S. lycopersicum* JASMONATE INSENSITIVE 1 (*jai1*) plants (mutated in the *coi1* gene) show decreased resistance to spider mites (Li *et al.*, 2004a). Also *N. attenuata* ir*coi1* plants grown in their natural environment showed a strong reduction in their resistance against insects (Paschold *et al.*, 2007). *A.*

thaliana jar1 mutant plants are more susceptible to the soil fungus *Pythium irregulare,* whereas *N. attenuata* ir*jar4/6* plants have reduced levels of several defense responses [e.g., nicotine and proteinase inhibitors (PIs)] as compared to WT plants. However, ir*jar4/6* plants differ in their response from those of plants silenced in NaLOX3 expression (as*lox3*) and therefore in total jasmonate accumulation (Wang *et al.*, 2008). A few hypotheses about the differences in the response to wounding and herbivory between JAR, COI1 and LOX deficient plants have been proposed. One of them is that the remaining levels of JA-Ile in *A. thaliana jar1-1* and *N. attenuata* ir*jar4*/6 plants (ca. 10% of WT levels) are still sufficient for the activation of defense responses (Suza and Staswick, 2008). Alternatively, other jasmonates than JA-Ile could interact with COI1 and JAZ proteins to activate defense responses.

The kinetics of jasmonate accumulation have been studied in a variety of plant species and large differences have been observed between them. In contrast to the OS-elicited JA and JA-Ile bursts in *N. attenuata* which wax and wane rapidly within 2h (Kang *et al.*, 2006), the wound-induced JA and JA-Ile burst in *A. thaliana* are much slower, requiring longer to attain maximum values (1.5 and 5h, respectively) and do not return to basal levels within 24h (Glauser *et al.*, 2008). Even among the same plant species there is considerate variation in leaf jasmonate accumulation; Miersch *et al.* (2008) showed that in the *S. lycopersicum* cultivar Lukullus, 12-OH-JA accumulation peaks at 4h while in the cultivar Castlemart it peaks at 8h or later, to attain concentrations twice as high as in the cultivar Lukullus.

Intact jasmonate signaling is required not only for the activation of defense responses in locally elicited leaves, but also for the propagation of the responses to unelicited systemic leaves. Using grafting experiments with the jasmonate biosynthesis *acx1* mutant and the jasmonate receptor *jai1* mutant, Li *et al.* (2002) elegantly showed that JA production is essential for the production, but not the reception of the systemic signal. Recently, studies performed with *N. attenuata* and *A. thaliana* plants have shown that JA-Ile is *de novo* biosynthesized in systemic leaves (Wang *et al.*, 2008; Koo *et al.*, 2009), and that very rapid events cause the systemic accumulation of JA (Glauser *et al.*, 2009). The relevance of these rapid increases in JA or JA-Ile on the activation of defense mechanisms in distal tissue however remains unknown.

Solanum nigrum is a wild, uncultivated Solanaceous plant that responds transcriptionally to both jasmonate treatment and competition (Schmidt, DD and Baldwin, IT, 2006). In a study comparing herbivore-induced transcriptional responses between *S. nigrum* and *N. attenuata*, it was shown that their transcriptional responses overlapped only by 10% (Schmidt *et al.*, 2005). In addition, the peptide systemin does not play a significant role in the induction of *S. nigrum*'s defense

responses, but potentially in the regulation of tolerance responses to herbivory (Schmidt, S and Baldwin, IT, 2006; Schmidt and Baldwin, 2009). Finally, it has also been demonstrated that a direct defense trait –a leucine aminopeptidase (LAP)– plays an important role in *S. nigrum*'s defense against Lepidopteran herbivores (Hartl *et al.*, 2008). Together, these results suggest that responses to herbivory in *S. nigrum* differ substantially from those in other plant species, and these differences prompted us to investigate jasmonate-mediated responses in this plant species. Hence for this study, *S. nigrum* plants silenced in three key steps of the jasmonate signaling cascade (biosynthesis, perception and conjugation) were produced and analyzed to determine the roles played by these genes in jasmonate biosynthesis, metabolism, and systemic signaling. Dramatic differences were observed among plants silenced in the expression of SnLOX3 (biosynthesis), SnCOI1 (perception), SnJAR4 (conjugation) and the WT, pointing to specific roles of each gene in the regulation of JA biosynthesis, metabolism and systemic signaling.

Material and Methods

Generation of transgenic lines and plant growth

Solanum nigrum L. inbred line Sn30 (Schmidt *et al.*, 2004) were used in all experiments, and were germinated and cultivated as described in Schmidt, S and Baldwin, IT (2006). Fragments of SnJAR4, SnLOX3 and SnCOI1 were identified using tobacco and *S. lycopersicum* sequences (accession numbers: AY254349, DQ359729 and AY423550), and used to create inverted-repeat constructs in the pSOL3 vector (Bubner *et al.*, 2006) (Fig. S2). *Agrobacterium*-mediated plant transformation with these inverted-repeat constructs was performed as described by Krügel *et al.* (2002). Hygromycin resistance, mediated by the *hptII* gene in the transformation vector, allowed homozygous lines to be selected. Insertion numbers were analyzed by Southern blotting using a PCR fragment of the *hptII* gene as a probe (Fig. S2). Single-insertion lines (line 1) of all genotypes were used in the experiments.

All experiments were performed with approximately 4 week-old plants that had just started flowering. Unless otherwise specified, "W+OS elicitation" refers to wounding plants by rolling a fabric pattern wheel three times on each side of the midvein and applying 20 µl 1:5 diluted *M. sexta* oral secretions and regurgitants (OS) to the resulting puncture wounds. The OS was collected from 3rd to 4th instar caterpillars which fed on WT *S. nigrum* plants. OS collections was performed by agitating the insect and collecting the regurgitate with a pipette from its oral cavity. W+W elicitation refers to the same procedure, replacing OS with double-distilled (dd)H_2O (See Fig S1).

Quantification of mRNA levels by QPCR

For transcriptional analysis, leaf samples were harvested after different times and immediately frozen in liquid nitrogen. Approximately 100 mg frozen leaf material was added to 2 ml SafeLock (Eppendorf) tubes containing two 5 mm steel beads, and homogenized in a Genogrinder Model 2000 at 500 strokes min^{-1}. Total RNA was isolated according to Schmidt *et al.* (2004), using a protocol based on TRIzol. cDNA synthesis was performed using 1 µg total RNA in a 20 µl reaction using an Invitrogen (Karlsruhe, Germany) kit, and following the manufactures' instructions. cDNA reactions were diluted ten times, and 10 ng (2 µl) was used as a template in a qPCR reaction. qPCRs were performed by SYBR-green analysis using the elongation factor 1 alpha (ELF1α) as an internal standard, with the protocol described in Gilardoni *et al.* (2010). Primers for qPCR analysis were: Sn*LOX3* Fw: GTGGCCCACGTTAGCTACTCC Rv: GCATGAGTGGTGGACGGTTA Sn*JAR4* Fw: TGAAGAAAATGGGGGAACAG Rv: TGACCGACAATAGGGACACA. SnCOI1 Fw: AGGAATCGCGTGACAGAGAC Rv: CCCAATCTTCCGGTATCAAA. Sn*ELF1α* Fw: GTTTCACTGCCCAGCTCATA Rv: TGGGCTTGGTGCCATCATC. All qPCR data was evaluated as described by Pfaffl (2002).

Labeled jasmonate and recovery experiments

For identification of herbivore-induced jasmonates, 45 ng [2,3-^{3}H]JA (ARC chemicals, Saint Louis, MO) were dissolved in 3µl MeOH, and mixed with either 17 µl H_2O containing 0.02% (v/v) Tween-20 or 1:5 diluted *M. sexta* OS, and applied to six rows of 4 cm-long puncture wounds, made with a fabric pattern wheel. To fully induce the leaf, the rest of the leaf was elicited with either W+W or W+OS treatment. After 3 h, the wounded lamina area treated with radioactive JA was harvested, flash-frozen in liquid N_2, ground to a fine powder and extracted three times with 1 ml ethyl acetate. Extracts were combined and evaporated to dryness under a continuous stream of argon, dissolved in 50 µl 70% (v/v) MeOH/ddH_2O and centrifuged for 10 minutes at 13,200 *g* to remove particles. For radio-HPLC analysis, radioactive extracts were run on an Agilent HPLC 1100 Series, using a gradient of solvent A (0.05% (v/v) formic acid/ H_2O) and solvent B (0.05% (v/v) formic acid/acetonitrile) starting with a linear gradient of 20% to 70% (v/v) solvent B for 20 min, 70% (v/v) solvent B for 5 min, and 20% (v/v) solvent B for 5 min. The extract was separated with an RP Sphinx column (C18 and propylphenyl stationary phase, 15%C, 250 × 4.6 mm, 5 µm particle diameter, Macherey-Nagel, Düren, Germany) with a flow of 1 ml min^{-1}. For radiodetection, a flow scintillation analyzer 500TR (Packard), using Ultima-Flo AP (Perkin Elmer, Jügesheim, Germany) scintillation liquid was used. Peak areas were integrated and their ratios calculated using the total amount of Becquerels in the chromatogram.

For the identification of peaks differentially induced by *M. sexta* OS, 22 µg [1,2-^{13}C]JA (Baldwin *et al.*, 1997) and 3 µg unlabeled JA were dissolved in 10 µl MeOH and mixed with 60 µl 1:5 diluted *M. sexta* OS and applied to one wounded ir*lox3* leaf. ir*lox3* plants treated with 25 µg unlabeled JA were used as controls. Three hours after elicitation, samples were harvested, ground in liquid N_2 and extracted three times with 10 ml ethyl acetate. Extracts were combined, reduced to dryness *in vacuo* by rotary evaporation, and reconstituted in 500 µl 70% (v/v) MeOH/ddH_2O and centrifuged for 10 minutes at 13,200 *g* to remove particles. Samples were subsequently fractionated by HPLC using the retention time (Rt) identified from the radio-analysis (12-OH-JA and JA were collected as controls). After fractionation, the solvent was evaporated under nitrogen and reconstituted in 70% (v/v) MeOH/ddH_2O. After centrifugation to remove particles, samples were injected in an LC-ESI-TOF (MicroToF, Bruker Daltonics, Bremen, Germany) in the negative mode connected to a HPLC system (Agilent HPLC 1100 Series) equipped with a Phenomenex Gemini NX 3 µm column (150 × 2 mm), using solvents A (ddH_2O 0.05% FA) and B (ACN 0.05% FA) as mobile phase. The gradient was first isocratic at 5% (v/v) solvent B for 2 min, followed by a linear gradient to 80% (v/v) solvent B for 28 min, 80% (v/v) solvent B for 6 min, and 5% (v/v) solvent B for 9 min, at a flow rate of 0.2 ml min^{-1}.

Jasmonate analysis

To study the accumulation of jasmonates, plants were W+OS and W+W elicited, and harvested after different times. Recovery experiments with JA or JA-Ile were performed by wounding plants as described above and applying 10 µg JA or JA-Ile, dissolved in 3 µl MeOH and added to 17 µl 1:5 diluted OS, to these wounds. After 4 h, tissue was harvested in liquid nitrogen, extracted and analyzed as follows. For jasmonate analysis, finely ground leaf material was extracted with 1 ml ethyl acetate containing 300 ng $^{2}D_2$-JA and 20 ng^{-1} JA-$^{13}C_6$-Ile as internal standard (IS). JA-$^{13}C_6$-Ile was synthesized as described by Kramell *et al.* (1988), using $^{13}C_6$-Ile (Sigma). Homogenates were centrifuged for 15 min at 13,200 *g*, the organic phase collected and evaporated to dryness. The dry residue was reconstituted in 0.4 ml 70% (v/v) MeOH/ddH_2O for analysis. Jasmonates were analyzed with an LC-MS/MS system (Varian 1200 Triple-Quadrupole-LC-MS system; Varian, Palo Alto, CA), 10 µl of the sample was injected onto a ProntoSIL® column (C18; 5 µm, 50 × 2 mm, Bischoff, Leonberg, Germany) connected to a precolumn (C18, 4 x 2 mm, Phenomenex, USA). As mobile phases 0.05% / 1% (v/v/v) formic acid/acetonitrile/water (solvent A) and 0.05% (v/v) formic acid/acetonitrile (solvent B) were used, starting with 15% (v/v) solvent B for 1.5 min, a linear gradient to 98% (v/v) solvent B for 3 min, 98% (v/v) solvent B for 8 min and 15% (v/v) solvent B for 2.5 min. Flow rates were: 0.4 ml min^{-1} for 1 min and 0.2 ml min^{-1} from 1 to 15 min. JA, JA-Ile, 12-OH-JA and 12-OH-JA-Ile were detected in the ESI negative mode and multiple reaction monitoring (MRM)

Limits of detection were established for JA and JA-Ile, and were 0.25 and 0.312 ng (amount on column), respectively (data not shown).

For JA-glucose, LC-(ESI)-MS/MS was not optimal for detection, so an atmospheric pressure chemical ionization (APCI) source was used to increase ionization efficiency. LC conditions only required a slight adjustment; the flow rate of the mobile phase was increased to 0.4 ml min^{-1}, with the same gradient conditions. APCI settings were: drying gas 12 PSI at 150°C, corona current 13 µA, APCI torch 350°C, nebulizing gas pressure 60 PSI, housing temperature 50°C and a shield voltage of -600V. For 12-OH-JA, ^{2}D-dihydro-JA was used as IS while JA-$^{13}C_6$-Ile was used for 12-OH-JA-Ile and JA-glucose. See Table S1 for details regarding ions and conditions used in the analysis.

Systemic signaling experiments

For systemic jasmonate analysis, local +1 leaves were either W+OS or W+W elicited and the vascularly connected systemic +5 leaves above the local leaf were harvested at different times after elicitation. JA and JA-Ile levels were quantified as described above. For ablation experiments, local +1 leaves were elicited by W+W or W+OS and ablated at the stem/petiole junction at different times after elicitation. Vascularly connected leaves at the +5 position above the wounded leaves were harvested after 48h and immediately frozen in liquid nitrogen. LAP levels were analyzed by isolating protein according to Jongsma (1993), protein concentration was analyzed using the Bradford assay. For LAP activity, 1.2 µg total protein was used in a 200 µl reaction mixture containing 0.05M tris-HCl pH 7.6, 0.05mM Mn and 2.5 mM L-Leu-β-naphtylamide. Reactions were incubated at 37°C and after 30 min the reaction was stopped with 40 µl 40% acetic acid, and absorption analyzed at 405 nm (Tecan Infinite M200 reader, Carlsheim, Germany). Blanks were measured by adding the same amount of protein to a stopped reaction mixture, measured at 405 nm and these blank values were subtracted from the values obtained after incubation. Three technical replicates were used for all LAP measurements.

Statistical analysis

All experiments were performed with 3 to 5 individual plants, representing biological replicates. Statistical calculations were performed with SPPS v. 17, a *log*-2 transformation was applied when data was not homoscedastic.

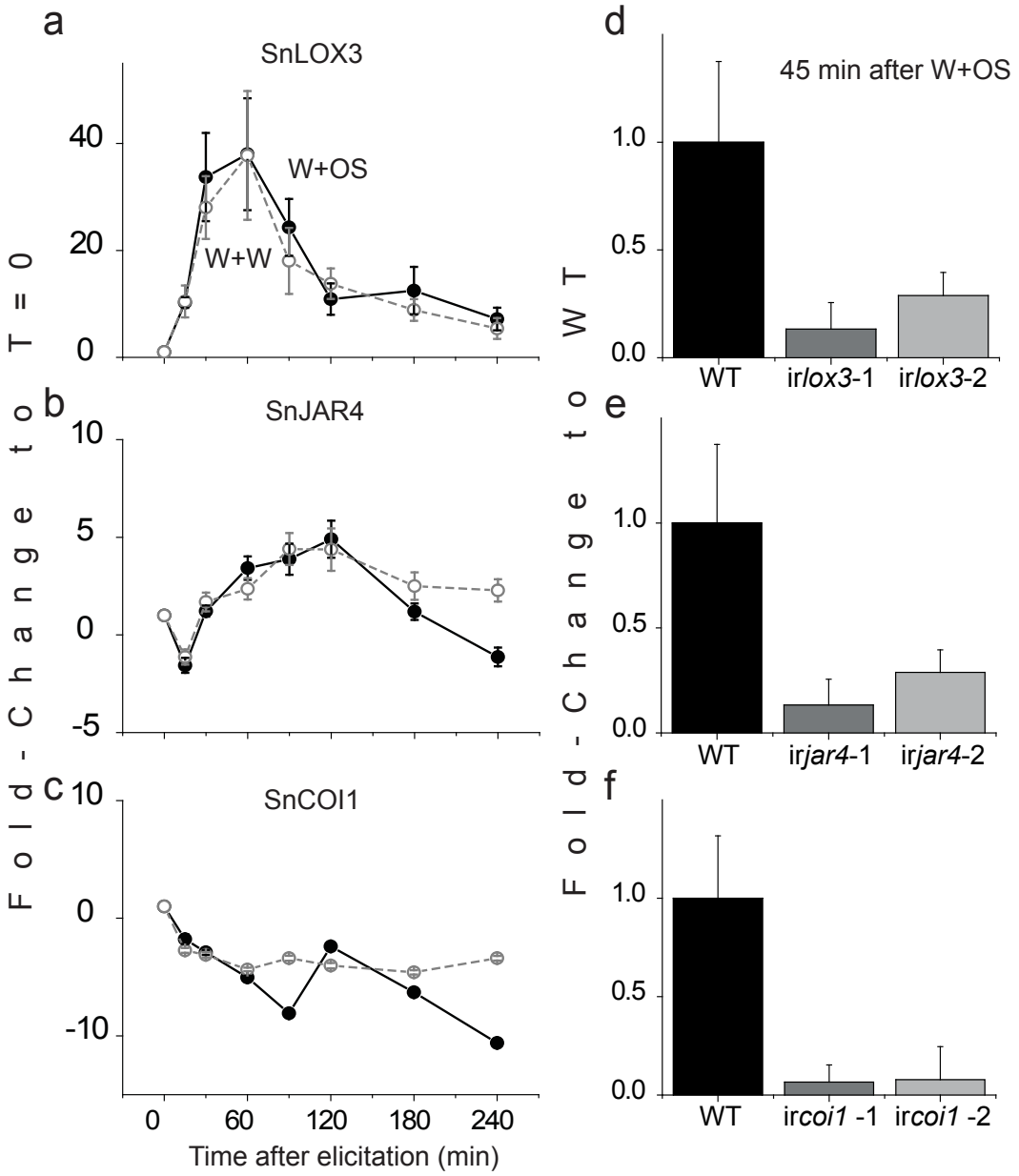

Figure 1. Analysis of sn*LOX3*, sn*JAR4* and sn*COI1* transcript levels and silencing efficiency of transgenic inverted-repeat lines. (**a,b,c**) WT plants were wounded by rolling a pattern wheel three times on each side of the midvein, and immediately applying 20 µl ddH_2O (W+W) (dashed line, open circles) or 1:5 diluted *M. sexta* oral secretions and regurgitants (W+OS) (solid line, closed circles) to the resulting puncture wounds. Tissue was harvested after different times, total RNA isolated and transcript levels quantified by qPCR. (**d,e,f**) Silencing efficiency of ir*lox3*, ir*jar4* and ir*coi1* plants. Plants were W+OS elicited and leaf tissues harvested after 45 min. Data is presented as fold-change to T=0 (**a,b,c**) or to WT levels (**d,e,f**). Experiments were performed with 4 to 5 biological replicates; error bars represent ± S.E.

Results

Generation of transgenic plants silenced in the expression of SnLOX3, SnCOI1 and SnJA4 genes

For the identification of *LOX*, *JAR* and *COI* genes involved in jasmonate biosynthesis and perception in *S. nigrum*, primers were designed based on the sequences of *N. attenuata* and *S. lycopersicum* Na*LOX3*, Na*JAR4*, and Sl*COI1*; the genes involved in jasmonate biosynthesis and perception in these plant species. A cDNA preparation from unelicited and OS-elicited *S. nigrum* leaf material was used as template for amplification of the corresponding homologues. After sequencing, the corresponding cDNA sequences were named accordingly Sn*LOX3*, Sn*JAR4* and Sn*COI1*. Accession numbers are HQ377507 (Sn*LOX3*), HQ377509 (Sn*JAR4*) and HQ377508 (Sn*COI1*).

The expression of these genes was first analyzed in a time series after leaf mechanical damage followed by application of either ddH_2O (W+W) or 1:5 diluted *M. sexta* OS (W+OS). Sn*LOX3* transcript levels showed a rapid, 40-fold induction after elicitation by both W+W and W+OS treatment (Fig. 1a), while SnJAR4 showed an initial, 1.3-fold downregulation, after which levels increased to 5-fold above control levels at 120 min, and returned to approximately basal levels after 4 h (Fig. 1b). Transcript levels of SnCOI1 showed a gradual downregulation, and after W+OS elicitation, levels were 10 times lower than basal levels after 4 h (Fig. 1c).

Transgenic *S. nigrum* plants harboring an inverted-repeat construct for each of the above mentioned genes were generated as described in Material and Methods. These lines were named ir*lox3*, ir*coi1* and ir*jar4* and two independently silenced lines for each gene were used in the experiments. Analysis of mRNA levels in leaves of mature plants by quantitative PCR showed that the two ir*lox3* lines (1 and 2) had 87% and 71% reduced transcript levels compared to the WT, while ir*jar4* (lines 1 and 2, respectively) had 86 and 85% and ir*coi1* (lines 1 and 2) had 93 and 92% reduced transcript levels compared to the WT (Fig. 1d,e,f). The vegetative growth and morphology of ir*lox3*, ir*coi1* and ir*jar4* plants was similar to that of WT plants. However, as described from other plant species (Li *et al.*, 2004b), ir*coi1* plants showed abnormal flower development [ir*coi1* flowers did not open, and after pollination the flower organs did not senesce, resulting in styles attached to the berries (Fig. S4)].

Identification of OS-induced jasmonates in S. nigrum leaves

Before profiling known jasmonates in the *S. nigrum* WT and silenced lines described above, we developed a system to identify jasmonates differentially induced after elicitation by W+OS treatment. First, 45 ng [2,3-^{3}H]JA were mixed with either 1:5 diluted *M. sexta* OS or water and applied to

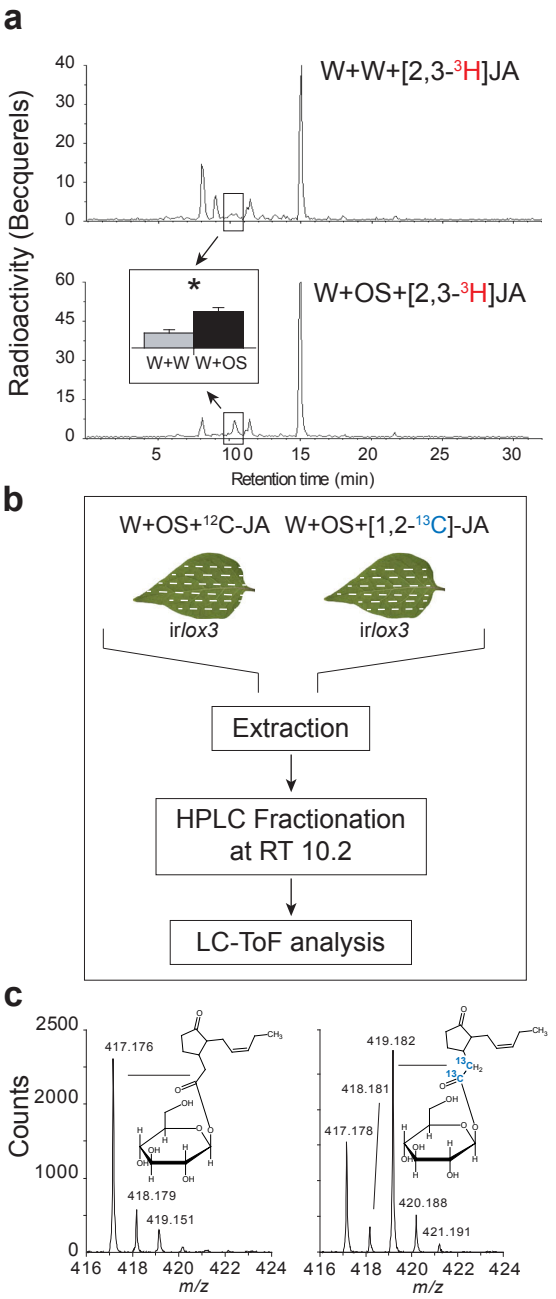

Figure 2. Identification of JA-glucose. (**a**) 45 ng [2,3-^{3}H]JA was mixed with either OS or ddH_2O containing 0.02% Tween-20, applied to wounded WT leaves, tissue extracted after 3h and radio-HPLC analysis performed. **Inset** shows peak integration of three biological replicates for the peak at 10.2 min, asterisk indicates a statistically significant difference; unpaired Student's *t*-test, $P<0.05$. (**b**) Rationale for purification of JA-glucose using [1,2-^{13}C]JA. ir*lox3* plants were used to avoid competition of the labeled substrates with the endogenous JA pools; preliminary experiments with WT plants the labeling efficiency was very low. Plants were wounded with a pattern wheel, and a mixture of 3/22 µg ^{12}/^{13}C JA was added to the resulting puncture wounds, this ratio was used to prevent a 100% ^{13}C labeling. Fractions corresponding to the chromatographic peak at 10.2 min in panel (**a**) were collected and analyzed by LC-TOF. (**c**), Mass spectra of fractions containing an enriched ^{12}/^{13}C-ratio. **Inset** shows the structure of JA-glucose.

wounded leaves. After 3h, leaf extracts were separated by TLC and several derivatives of [2,3-^{3}H]JA were detected (Fig. S2). For quantification, leaf samples were also analyzed by reverse phase radio-HPLC (Fig. 2a) and several peaks showed differential accumulation in W+OS+ [2,3-^{3}H]JA compared to W+W+ [2,3-^{3}H]JA samples (e.g., peak with Rt: 10.3 min was 3-fold higher in W+OS+ [2,3-^{3}H]JA than in W+W+ [2,3-^{3}H]JA treated leaves; Fig. 2a). The Rt of the radiolabeled compounds was used to identify these differentially accumulating jasmonates as follows: 22 µg of [1,2-^{13}C]JA were mixed with 3 µg of unlabeled JA (to prevent complete labeling) dissolved in 1:5 diluted *M. sexta* OS and applied to a wounded ir*lox3* leaf. For these experiments, we used ir*lox3* plants to avoid competition of the labeled substrates with the endogenous JA pools; in preliminary experiments with WT plants the labeling efficiency was very low (data not shown).

As a control, an ir*lox3* leaf was treated with 25 µg of unlabeled JA dissolved in OS. Extracts were fractionated by preparative HPLC using the Rts from the radio-chromatograms as reference for elution times, and the fractions were analyzed by LC-TOF using the rationale that a molecule with a backbone of $C_{20}O_3$ would typically generate an M+2 isotopic peak sized 3.06 % of the monoisotopic

peak (M) and that $^{13}C_2$ incorporation would change this isotopic distribution considerably. Plants treated with unlabeled JA were used to control for other incorporations (for example, a chloride atom) that would also cause a significant M+2 shift. Chromatograms were screened for isotope distribution patterns that had a significant M+2 shift in the labeled extract, but a normal isotope distribution in controls.

A chromatographic peak with a monoisotopic *m/z* 417.176 showed an *m/z* 419.182 M+2 isotopic peak intensity that was 183% of the monoisotopic peak. Both the monosiotopic and the M +2 masses corresponded exactly to the formiate adducts of JA-glucose (417.177 and 419.183 Da, respectively) (Fig. 2c), while the formiated molecule showed a neutral loss of 46.004, resulting in *m/z* 371.172, corresponding to the $[M\text{-}H]^-$ of JA-glucose. A further neutral loss of 163.05 corresponded to the sugar moiety, and resulted in an ion with *m/z* 209.11 and *m/z* 211.11, corresponding to both labeled and unlabeled JA. (Fig. 2c). JA-glucose was synthesized according to Qian *et al.* (2004) and its Rt was identical to the isolated compound (data not shown). Two additional fractions collected by preparative HPLC were analyzed using the same method and these fractions contained 12-OH-JA and 12-OH-JA-Ile (data not shown). Additional jasmonates could not be identified by this method, most likely due to low levels of accumulation.

Jasmonate profiling in S. nigrum leaves after wounding and OS elicitation

Leaves of *S. nigrum* WT plants were analyzed for JA, JA-Ile, JA-amino-acid conjugates, 12-COOH-JA, 12-COOH-JA-Ile, 12-OH-JA, 12-OH-JA-Ile, JA-glucose and 12-SO_4-JA at different times after elicitation by either W+W and W+OS treatments. Among these jasmonates, only JA, JA-Ile, 12-OH-JA, 12-OH-JA-Ile and JA-glucose accumulated to detectable levels (Fig. 3).

In WT plants, JA levels were induced by W+W and reached ~400 ng g^{-1} FW after 30 min, remaining relatively constant up to 180 min when they started to decline (Fig. 3a-I). During the first 90 min after W+OS elicitation, JA levels accumulated to similar amounts to those in wounded leaves, however a second JA burst peaking at 120 to 180 min and reaching ~2,000 ng g^{-1} FW was specifically induced by OS (Fig. 3a-I). Similarly, JA-Ile levels peaked rapidly at 30 min to 150 ng g^{-1} FW after both W+W and W+OS, with a second burst induced only by OS but smaller in magnitude compared to the first JA-Ile peak (Fig. 3b-I). In contrast to JA and JA-Ile, 12-OH-JA, 12-OH-JA-Ile did not show a rapid accumulation, but their levels started to increase slowly 90 min after elicitations. Consistent with the differential accumulation of JA after OS elicitation, the levels of these hydroxylated jasmonates were higher after this treatment compared to W+W (Figs. 2c-I and 2d-I). JA-glucose also followed the JA burst, W+OS increased levels 3 to 4-fold over W+W elicitation (Fig 3e-I).

In ir*lox3* plants, JA levels were strongly reduced to less than 2% of WT levels and similar reductions were detected for 12-OH-JA, 12-OH-JA-Ile and JA-glucose (<1% of WT levels) (Fig. 3, a-II,c-II, d-II and 3-II). In contrast, the first JA-Ile burst only showed a 70% reduction compared to WT levels and the second JA-Ile OS-elicited burst could not be detected in these plants (Fig. 3b-II).

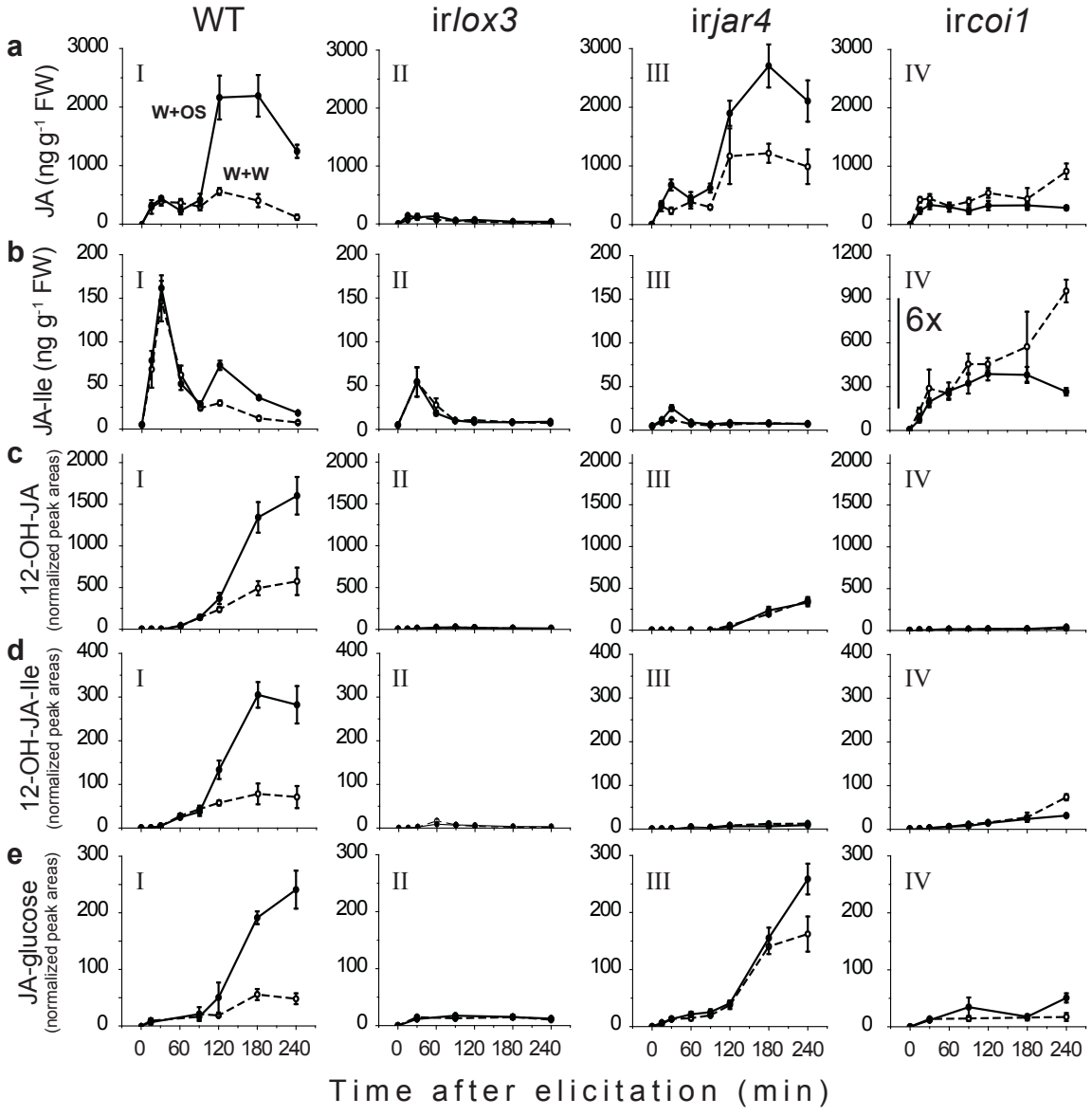

Figure 3. Jasmonate profiles of *S. nigrum* WT, ir*lox3* , ir*jar4* and ir*coi1* plants after W+W and W+OS elicitation. Plants were (W+W) (dashed lines, open circles) or (W+OS) (solid lines, closed circles) elicited and leaf tissue was harvested after different times, extracted and analyzed by LC-MS/MS. Jasmonates analyzed were (**a**) jasmonic acid (JA), (**b**) jasmonyl-isoleucine (JA-Ile) (**c**) 12-hydroxy-jasmonate (12-OH-JA) (**d**) 12-hydroxy-jasmonyl-isoleucine (12-OH-JA-Ile) (**e**) jasmonyl-*O*-glucose (JA-glucose). Panel numbers (**I-IV**) refer to the kinetics in WT, ir*lox3*, ir*jar4* and ir*coi1*, respectively. Note the 6-fold increase of the axis in panel **b-IV**. Experiments were performed with 3 to 5 biological replicates, error bars represent ± S.E.

In ir*jar4* plants, JA levels were induced to higher than WT levels after both W+W and W+OS (Fig. 3a-III). Interestingly, in these plants, W+W also induced a second JA burst which was absent in WT plants (Fig. 3a-I). As expected, JA-Ile levels were strongly reduced in ir*jar4* plants and only 15% of WT levels could be detected 30 min after induction (Fig. 3, b-III). Although JA-Ile accumulated to low levels in ir*jar4* plants, W+OS induced a two-fold increase in JA-Ile levels compared to W+W (Fig. 3 b-III). 12-OH-JA and 12-OH-JA-Ile levels were reduced in ir*jar4* plants to 20% and 3% of WT levels, respectively (Fig. 3c,d-III). Strikingly, W+OS did not induced an increased accumulation of these hydroxylated JA forms in ir*jar4* plants, even though the OS elicited JA burst was more pronounced in these plants than in the WT (compare Figs. 2a-I, 2a-III, 2c-I and 2c-III). JA-glucose levels were unchanged after W+OS elicitation, but W+W levels were augmented (~3-fold) in ir*jar4* plants (Fig 3e-III).

ir*coi1* plants failed to produce the OS-induced JA burst however JA accumulated to levels similar to WT plants after W+W. Importantly, JA levels did not drop after 180 min of W+W but even increased to ~800 ng g^{-1} FW (eight times more than in WT plants at 240 min; Fig. 3, a-IV). Moreover, in contrast to the rapid decrease in JA-Ile 30 min after the treatments in WT plants, JA-Ile accumulated continuously in ir*coi1* plants after W+W to reach ~1,000 ng g^{-1} FW and ~250 ng g^{-1} FW after W+OS at 240 min (Fig. 3, b-IV; note 6-fold change in y-axis). These levels corresponded to an 80- and 12-fold increase in JA-Ile levels compared to WT plants 240 min after W+W and W+OS elicitations, respectively. Similar to ir*lox3* plants, the levels of 12-OH-JA and 12-OH-JA-Ile were reduced to 2 and 8% of WT levels in ir*coi1* plants, respectively (Fig. 3, c-IV and d-IV). JA-glucose accumulations were also 8-9 fold reduced in ir*coi1* plants (Fig. 3e-IV).

Regulation of JA, JA-Ile, 12-OH-JA and 12-OH-JA-Ile accumulation in leaves

Turnover of JA-Ile has previously been shown to be a COI1-dependent mechanism in *N. attenuata*, most likely by the COI1-dependent activation of genes involved in its modification (Paschold *et al.*, 2008). To test if reduced turnover could explain the higher JA-Ile levels in *S. nigrum* ir*coi1* plants compared to the WT, especially after W+W elicitation (Fig. 3a-IV), 500 ng of JA-$^{13}C_6$-Ile were applied onto WT and ir*coi1* wounded leaves and the samples harvested at different times and analyzed by LC-MS/MS. The results showed that the decay rates of JA-$^{13}C_6$-Ile were similar between WT and ir*coi1* plants (Fig. 4a), suggesting that JA-Ile turnover was not substantially affected in ir*coi1* plants.

To test if increased JAR4 activity could instead explain the high JA-Ile levels, WT, ir*coi1*, ir*lox3* and ir*jar4* plants were elicited by W+OS which was supplemented with 10 µg of JA and JA-Ile levels quantified after 240 min of the treatment.

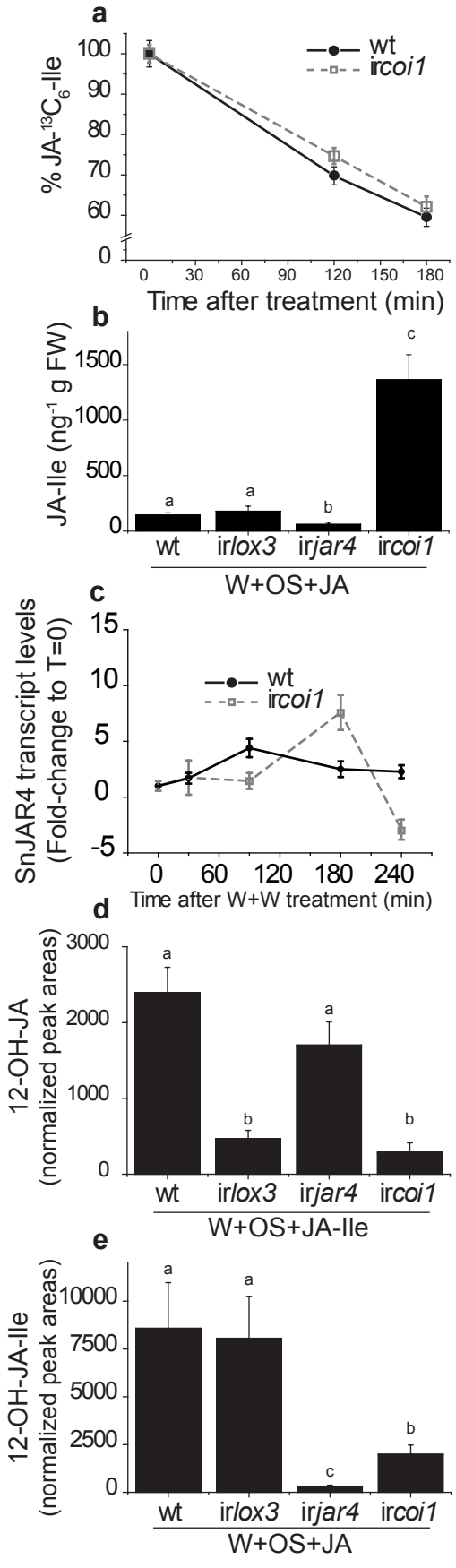

Figure 4. Regulation of jasmonate metabolism by SnCOI1 and JA-Ile. (**a**) JA-Ile metabolism reveals that ir*coi1* plants do not differ from WT in JA-Ile metabolism. 500ng JA-$^{13}C_6$-Ile was applied to wounded ir*coi1* (dashed line open squares) and WT (solid line, closed circles) leaves, samples harvested after different times and levels of JA-$^{13}C_6$Ile were quantified. To levels were set to 100%. (**b**) JA-Ile accumulation after supplementing W+OS with JA; plants were treated by W+OS supplemented with 10 µg JA, treated leaves harvested after different times and analyzed for JA-Ile. Different letters denote a significant difference (univariate ANOVA $F_{3,3}$ = 61.4, P<0.05 followed by a Scheffé *post-hoc* test). (**c**) Sn*JAR4* transcript levels in ir*coi1* and WT plants. Leaves were harvested at different times after W+W elicitation, and mRNA levels of Sn*JAR4* quantified by qPCR. (**d**) 12-OH-JA accumulation is dependent on SnCOI1 and JA-Ile. Plants were treated with W+OS supplemented with 10 µg JA-Ile, treated leaves harvested after 4h and 12-OH-JA levels analyzed. Different letters denote a significant difference (univariate ANOVA $F_{3,3}$=17.2, P<0.001 followed by a Scheffé *post-hoc* test). (**e**) 12-OH-JA-Ile accumulation is dependent on JA biosynthesis, SnJAR4 activity and SnCOI1. Plants were treated with W+OS supplemented with 10 µg JA, treated leaves harvested after 4h and 12-OH-JA-Ile levels analyzed by LC-MS/MS. Different letters denote a significant difference (univariate ANOVA $F_{3,3}$ = 36.1, P<0.001 followed by a Scheffé *post-hoc* test). All experiments were performed with 4 to 5 replicates, all error bars represent ± S.E.

JA-Ile levels were 10-fold higher in ir*coi1* plants than in WT plants (Fig. 4b), suggesting that higher JAR4 enzyme activity in ir*coi1* plants could be responsible for the high JA-Ile levels. *In planta*, however, this would require a constant supply of JA for the JAR4 catalyzed reaction and this is indeed consistent with the higher and constant levels of JA (~800 ng g^{-1} FW) observed in ir*coi1* plants after W+W (Fig. 3a-IV). Transcriptional analysis of SnJAR4 in ir*coi1* plants showed that SnJAR4 levels were only higher after 3 h, indicating that SnCOI1 does not regulate JAR4 on a transcriptional level (Fig. 4c).

To test if the reduced 12-OH-JA levels in ir*jar4* plants (Fig. 3c-III) were related to the reduced levels of JA-Ile,

WT and ir*jar4* plants were treated with OS+10 µg JA-Ile and the 12-OH-JA levels were quantified in leaves. The results showed that after JA-Ile supplementation, 12-OH-JA levels were restored in ir*jar4* plants to WT levels whereas they were not restored in JA-Ile-supplemented ir*lox3* and ir*coi1* leaves (Fig. 4d).

In contrast, 12-OH-JA-Ile levels were restored by supplementing JA in ir*lox3* wounded leaves but not when ir*jar4* or ir*coi1* plants were supplemented with JA (Fig. 4e).

Activation of systemic signaling in S. nigrum leaves depends on OS elicitation and jasmonate biosynthesis and perception

Because the accumulation of the identified jasmonates showed different patterns over time, we dissected the significance of this temporal regulation by investigating the activation of systemic signaling. First, we were interested in the systemic accumulation of jasmonates, so local +1 leaves were either W+W or W+OS elicited and JA and JA-Ile quantified in the vascularly connected +5 systemic leaves (Fig. 5a,b). In systemic leaves of WT plants, JA amounts remained between ~10 to 20 ng g^{-1} FW within 60 min after W+W and W+OS elicitation, and they increased by 4-fold after 240

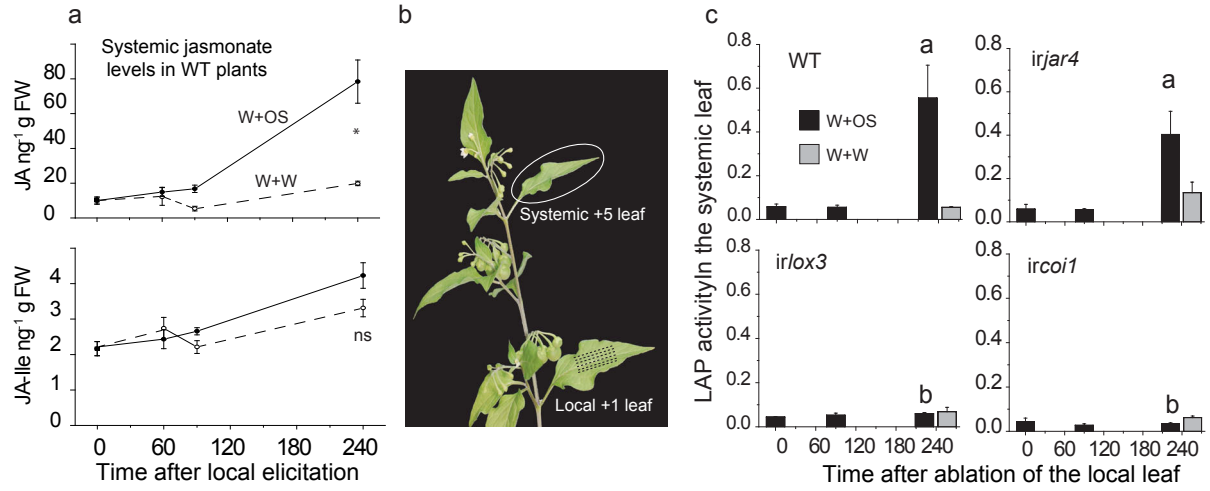

Figure 5. Systemic accumulation of jasmonates and LAP activity after local elicitation. (**a**) Systemic JA and JA-Ile accumulation after local treatments; +1 leaves were elicited by either W+W (open circles, dashed lines) and W+OS treatments (closed circles, solid lines) after which +5 vascularly connected systemic leaves were harvested after different times and JA and JA-Ile quantified by LC-MS/MS. Asterisk indicates a significant treatment effect, unpaired Student's *t*-test, $P<0.05$. ns = not-significant. (**b**) Photograph showing the experimental set-up, the lower +1 leaf was elicited, and the upper, connected +5 leaf harvested. (**c**) Local leaves were W+W or W+OS elicited and leaves including petioles ablated at 0, 90 or 240 min after treatment. The vascularly connected, +5 systemic leaf was harvested 48h after treatment for leucine aminopeptidase activity (LAP) analysis. Different letters denote significant differences (univariate ANOVA $F_{3,3} = 23.1$, $P<0.001$ followed by a Scheffé post-hoc test). All experiments represent averages of 4 to 5 replicates, all error bars represent ± S.E.

min of W+OS but not W+W treatment. JA-Ile levels were slightly induced in systemic leaves by both treatments from ~2 to 3.5 ng g^{-1} FW (Fig. 5a).

To investigate the influence of local jasmonate accumulation on the activation of systemic defenses, local +1 leaves were elicited by W+W or W+OS and ablated from the petiole's base after different times. Systemic, vascularly connected +5 leaves were harvested 48 h after local induction and analyzed for the activity of the defense protein leucine aminopeptidase (LAP). In WT plants, LAP activity in systemic leaves was induced only after 240 min of W+OS, but not after W+W elicitation (Fig. 5c), indicating that the systemic signal required between 90 and 240 min to leave the local leaf –a time period that corresponded with the induction of the second JA burst in treated leaves after W +OS elicitation (Fig. 3a-1). In ir*lox3* and ir*coi1* plants, no induction of LAP activity in systemic leaves after elicitation occurred, while in ir*jar4* plants the systemic response in LAP activity attained 75% of WT levels (Fig. 5c).

Discussion

OS elicitation induces a second burst of JA in S. nigrum leaves

Profiling of jasmonates after wounding and *M. sexta* OS elicitation in *S. nigrum* leaves revealed that JA accumulates in a biphasic manner after OS elicitation; the immediate JA accumulation to 400 ng g^{-1} FW is followed by a 3 to 4-fold increase after 90 min. The amplitude of the OS-elicited JA bursts were not reflected in the JA-Ile bursts, where both W+W and W+OS treatments induced a large and similar peaks at 30 min, and only W+OS elicited a smaller second burst peaking after 90 min. These JA and JA-Ile bursts differ from those in *N. attenuata* and *N. sylvestris* leaves, in which *M. sexta* OS elicitation triggers single and temporally coordinated JA and JA-Ile bursts which reach maximum levels within 60 min (McCloud and Baldwin, 1997; Ziegler *et al.*, 2001; Kang *et al.*, 2006; Kallenbach *et al.*, 2010).

As expected, JA accumulation was completely dependent on Sn*LOX3* expression; ir*lox3* plants accumulated less than 2% of JA, 12-OH-JA and 12-OH-JA-Ile levels than in the WT, indicating that this LOX is the major isoform involved in jasmonate biosynthesis in *S. nigrum*. Interestingly however, JA-Ile levels were reduced only by 70% in ir*lox3* leaves, suggesting that the largest fraction of the JA produced in ir*lox3* leaves was used for the biosynthesis of JA-Ile –reflecting a metabolic priority for the synthesis of this molecular signal. Consistent with the positive transcriptional feedback that regulates most of the JA biosynthesis genes in different plant species, SnLOX3 transcript levels were induced by wounding and OS elicitation (Fig. 1a). SnJAR4 was initially downregulated after W+W and W+OS treatment, showing that JAR4 activity is regulated on a different level (Fig. 1b). A

differential JA and JA-Ile accumulation between wounding and OS elicitation could be observed in ir*jar4* plants as early as 30 min after the treatments, showing that OS perception and signaling occurs rapidly in *S. nigrum* leaves. Because differences in JA and JA-Ile accumulation after W+W and W+OS in WT leaves were observed only after 90 min of the stimuli, the results suggested that JA-Ile mediated mechanisms may prevent this differential induction to occur in WT leaves. Moreover, in ir*jar4* plants, W+W induced a second JA burst that was undetectable in WT after this treatment (Fig. 3 A-III), suggesting that a JA-Ile mediated mechanisms may also inhibit the production of the second JA burst after wounding in WT plants. This second OS-induced JA burst was however, dependent on Sn*COI1*, suggesting that a jasmonate different from JA-Ile may be responsible for positively regulating this burst by a *COI1*-dependent mechanism.

JA-Ile accumulation is under the negative control of SnCOI1

In *S. nigrum*, JA-Ile production was deregulated in ir*coi1* leaves, indicating that, similar to NaCOI1 in *N. attenuata* leaves (Paschold *et al.*, 2008), SnCOI1 negatively controls JA-Ile accumulation. In contrast to *N. attenuata* ir*coi1* plants, where reduced JA-Ile turnover leads to a delay and enhanced accumulation of JA-Ile (Paschold *et al.*, 2008), *S. nigrum* ir*coi1* plants lost completely the WT pattern of JA-Ile accumulation and produced JA-Ile constantly after W+W treatment for a period of at least 240 min (Fig. 3b-IV). This accumulation was accompanied by a constant supply of JA, as reflected by its constant levels during the 240 min period after the initial wounding (Fig. 3a-IV), and by an increased capacity to synthesize JA-Ile (i.e. higher JAR4 activity) when leaves were supplemented with exogenous JA (Fig. 4b). Thus, although COI1 negatively controls JA-Ile levels in *S. nigrum* and *N. attenuata*, the mechanism utilized by these plant species is different. However, in both species COI1 probably negatively regulates JA-Ile in order to reduce the active signal after perception.

SnCOI1 and SnJAR4 differentially promote the metabolism of hydroxylated and glucosylated forms of JA

Hydroxylated forms of JA were first discovered as tuber-inducing compounds, and 12-OH-JA is also known as "tuberonic acid" (Koda and Okazawa, 1988). Only relatively recently has their pattern of accumulation in response to wounding and their role in the wound response been studied (Miersch *et al.*, 2008). 12-OH-JA is present in a variety of plant species and in different plant organs, and it has been proposed to play a role as a negative regulator of the expression of JA biosynthetic genes (Miersch *et al.*, 2008). In *S. nigrum*, 12-OH-JA accumulation was dependent on SnCOI1 and SnJAR4, indicating that these two genes play a role in the regulation of JA hydroxylation (Fig. 3 c-II

and c-IV). 12-OH-JA levels could be recovered in ir*jar4* but not in ir*coi1* plants when wounded leaves were supplied with JA-Ile, demonstrating that 12-OH-JA formation is dependent on both JA-Ile signaling and COI1 perception (Fig. 4d).

12-OH-JA-Ile was first isolated from *S. lycopersicum* and *A. thaliana* leaves (Guranowski *et al.*, 2007), and it was shown to accumulate slower after the wound response than both JA and JA-Ile in *A. thaliana* plants (Glauser *et al.*, 2008). Similar to 12-OH-JA, accumulation of 12-OH-JA-Ile was reduced in ir*coi1* and ir*jar4* plants, showing that SnCOI1 and SnJAR4 also regulates the 12-hydroxylation of JA-Ile.

Using [2,3-^{3}H]JA and [1,2-^{13}C]JA, we were able to identify JA-glucose as a differentially OS-induced jasmonate in *S. nigrum*. JA-glucose was previously identified in tobacco bright yellow-2 (BY-2) cells after JA feeding (Qian *et al.*, 2004) and it was also detected in wounded *A. thaliana* leaves (Glauser *et al.*, 2008). In WT *S. nigrum* leaves, JA-glucose accumulated gradually for 240 min after W+W and W+OS, reflecting the levels of JA (Fig. 3e-I). JA-glucose levels were strongly reduced in ir*coi1* and ir*lox3* leaves, indicating that, similar to JA 12-hydroxylation, JA-glucosidation is also a SnCOI1-regulated process. Strikingly, JA-glucose levels were not reduced in ir*jar4* leaves but rather augmented by W+W compared to WT levels, reflecting the increased JA levels observed in these plants after W+W elicitation (Fig. 3 a-III). Thus, the results showed that SnCOI1 and SnJAR4 similarly control the hydroxylation of JA and JA-Ile, while differentially regulating the glucosidation of JA.

Induction of systemic defense gene activation depends on the second OS-elicited JA burst in S. nigrum

Because of the differential pattern of jasmonate accumulation between wounded and OS-elicited leaves, we investigated the relevance of this pattern in plant defense by ablating the local leaf at different time points after either W+W or W+OS elicitation and analyzing the levels of LAP activity (Hartl *et al.*, 2008) in vascularly connected systemic leaves (Fig. 5b). In WT *S. nigrum* plants, the signal that elicits systemic LAP activity requires more than 90 min to leave the local leaf and requires OS elicitation; no induction of systemic LAP activity was observed after W+W elicitation. Induction of systemic LAP activity was completely dependent on local jasmonate production and perception; ir*coi1* and ir*lox3* plants were impaired in the systemic induction of LAP activity (Fig. 5c). In contrast, induction of systemic LAP activity was only partially reduced in ir*jar4* compared to WT plants (Fig. 5c). Because ir*lox3* plants still accumulated 30% of JA-Ile levels in WT leaves (versus 15% in ir*jar4* plants), the results suggested that local JA-Ile accumulation was not essential for the generation of a systemic signal. The time required for the systemic signal to leave the leaf and its dependence on OS

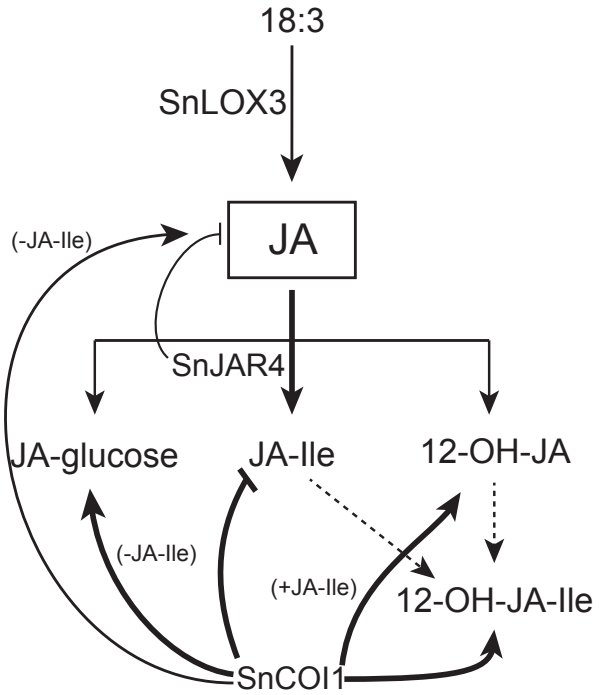

Figure 6. Schematic overview of jasmonate metabolism in *Solanum nigrum* SnLOX3 is essential for the biosynthesis of JA, which is metabolized into JA-Ile, JA-glucose and 12-OH-JA albeit with different kinetics. 12-OH-JA-Ile can either be synthesized from JA-Ile or 12-OH-JA-Ile. SnJAR4 is responsible for the conjugation of JA into JA-Ile, and negatively regulates JA. SnCOI1 strongly regulates 12-OH-JA accumulation in a JA-Ile dependent manner (+JA-Ile) but JA-glucose in a JA-Ile independent (-JA-Ile) manner, while JA-Ile accumulation is under strong negative regulation of COI1. JA accumulation is also regulated by SnCOI1, but this effect is weak and JA-Ile independent. Solid lines present biosynthetic pathways, line thickness is related to the strength of the regulation and dotted lines indicate putative pathways.

elicitation strongly suggested that the differential pattern of jasmonate production in *S. nigrum* leaves after OS perception is essential for the generation of the systemic signal.

Our results were consistent with grafting studies performed in tomato (Li *et al.*, 2002; Li *et al.*, 2005); jasmonate accumulation in the local leaf is essential for activation of the systemic signal. Studies in *A. thaliana* have shown that systemic JA and JA-Ile accumulations occur very rapidly (Glauser *et al.*, 2009; Koo *et al.*, 2009). In *S. nigrum*, basal JA levels were ca. 20 ng g^{-1} FW and increased to ca. 80 ng g^{-1} FW after W+OS, levels 40 times lower than in elicited leaves. JA-Ile only accumulated to low levels (2 to 3 ng g^{-1} FW) and it was not differentially induced by OS (Fig. 5a). Considering that both the systemic induction of LAP activity, and the accumulation of systemic JA required more than 90 min to leave the locally elicited leaf (Fig. 5a,c) suggested that the same signal activates both responses.

We present a schematic model in Fig. 6 that summarizes the regulation of jasmonate signaling and metabolism in *S. nigrum* leaves. OS elicitation induces a second JA burst that is strongly correlated with the generation of a systemic signal in local leaves and in the subsequent systemic induction of a defense trait (i.e. LAP) in WT *S. nigrum* plants. Future work will focus on the understanding of the mechanisms acting on the activation of jasmonate biosynthesis after OS perception, and in the generation of a systemic signal.

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SUPPLEMENTARY MATERIAL

Table SI. List of ion transitions and parameters used for analysis of compounds by LC-MS/MS.

Name of analyte	Molecular ion [M-1]	Fragment ion	Capillary CID	Collision energy
JA	209	59	-35V	12V
$^{2}D_{2}$-dihydro-JA	213	59	-35V	12V
JA-Ile	322	130	-45V	18V
JA-$^{13}C_{6}$-Ile	328	136	-45V	18V
12-OH-JA	225	59	-35V	12V
12-OH-JA-Ile	338	130	-45V	18V
JA-glucose (form. Adduct)	417	209	-35V	18V

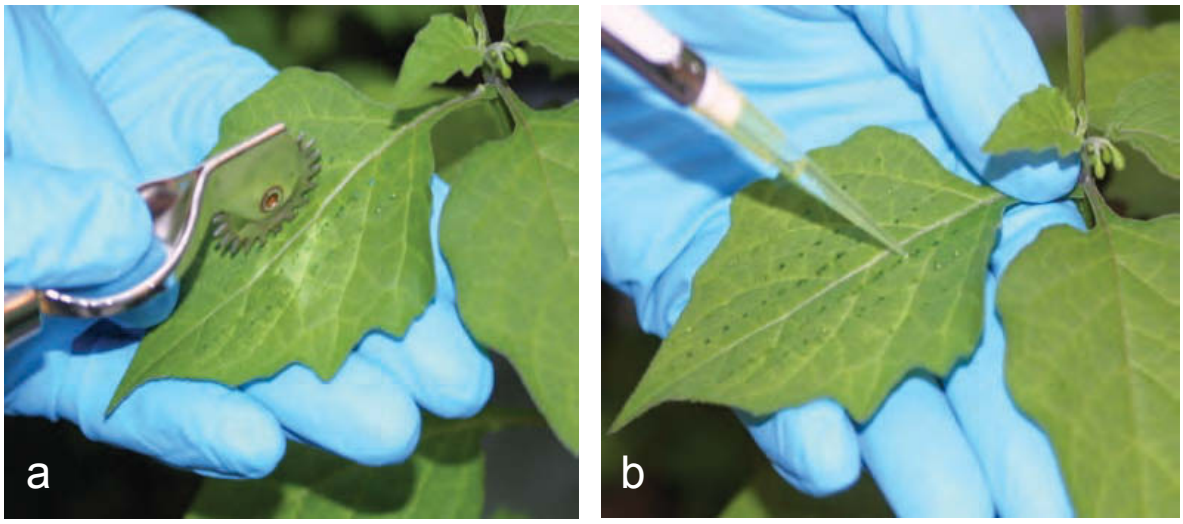

Figure S1. Photographs of the W+W and W+OS elicitation procedure.
(a) Leaves were wounded by rolling a pattern wheel over them, creating a row of puncture wounds. (b) ddH_2O or 1:5 diluted *M. sexta* oral secretions were subsequently applied to these wounds with a pipette.

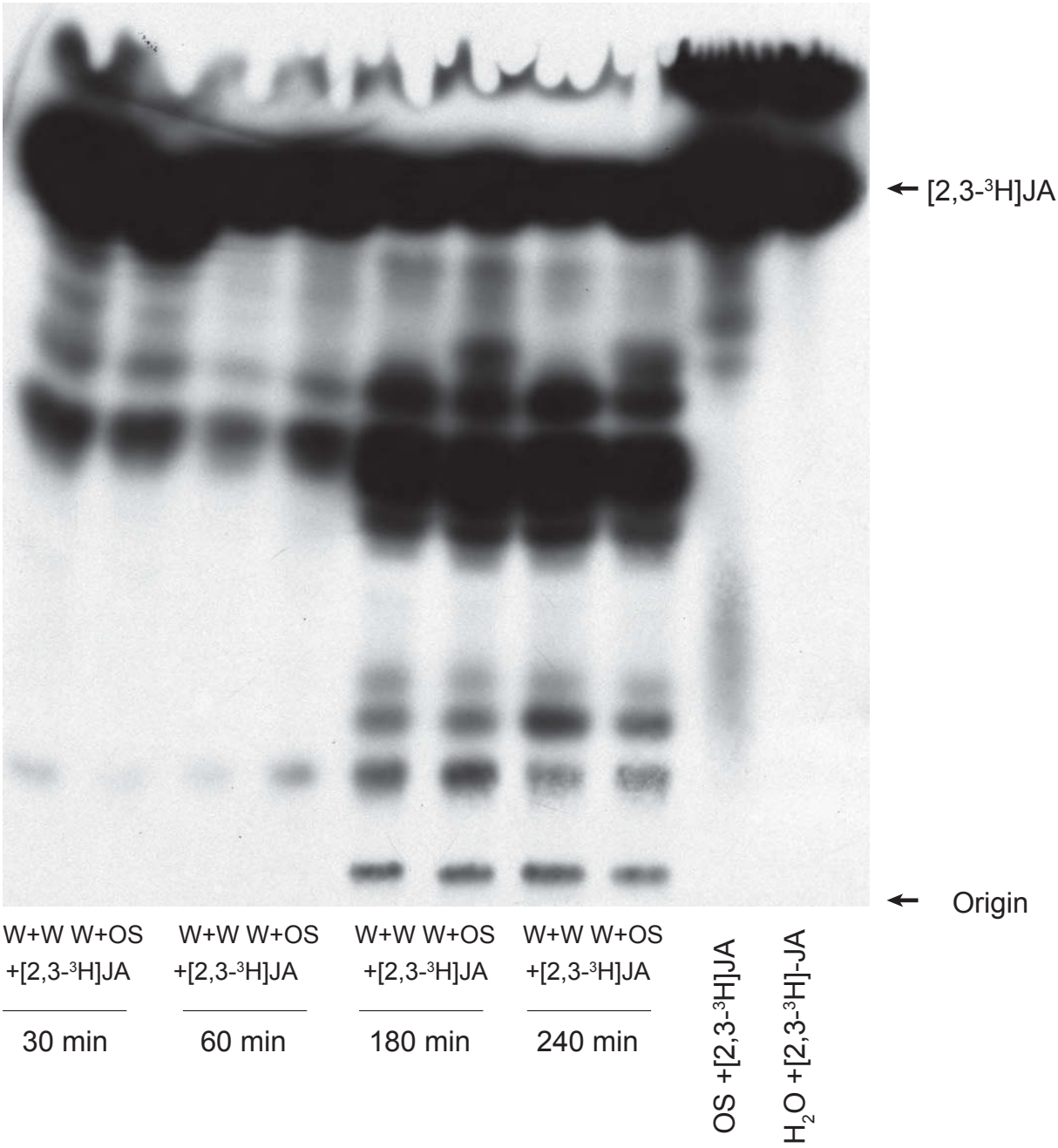

Figure S2. [2,3-^{3}H]-JA metabolism on WT plants after W+W and W+OS elicitation.
Leaves were wounded with a fabric pattern wheel and 45 ng [2,3-^{3}H]JA dissolved in either 1:5 diluted *M. sexta* OS, or ddH_2O containing 0.02% (v/v) Tween-20 were applied to the wounded leaves. The total treated area was harvested after different times, ground to a fine powder and extracted three times with 1 ml ethyl acetate. Extracts were evaporated and spotted on a silica gel-60 TLC. The mobile phase was 55/35/10 (v/v/v) chloroform/ethyl acetate/formic acid. TLCs were sprayed with En^{3}Hance spray (Perkin Elmer), exposed to X-ray film for 10 days at -80 °C and developed.

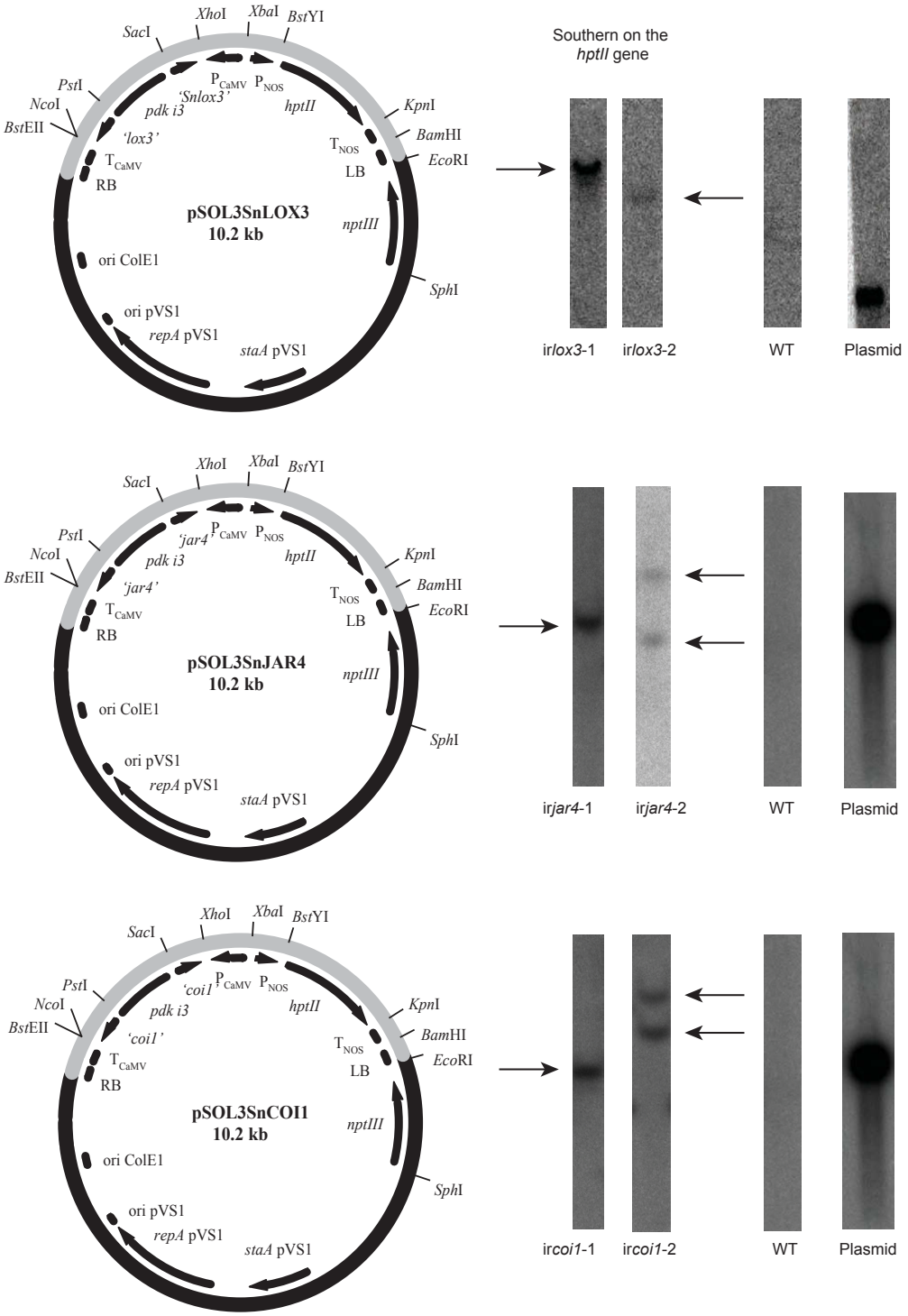

Figure S3. Plasmids used for plant transformation and Southern blots of transgenic lines. Vector maps of the plasmids for plant transformation. For Southern blots, 30 µg genomic DNA was digested with EcoRI, blotted and hybridizations were performed with a probe corresponding to the *hptII* gene, arrows indicate bands.

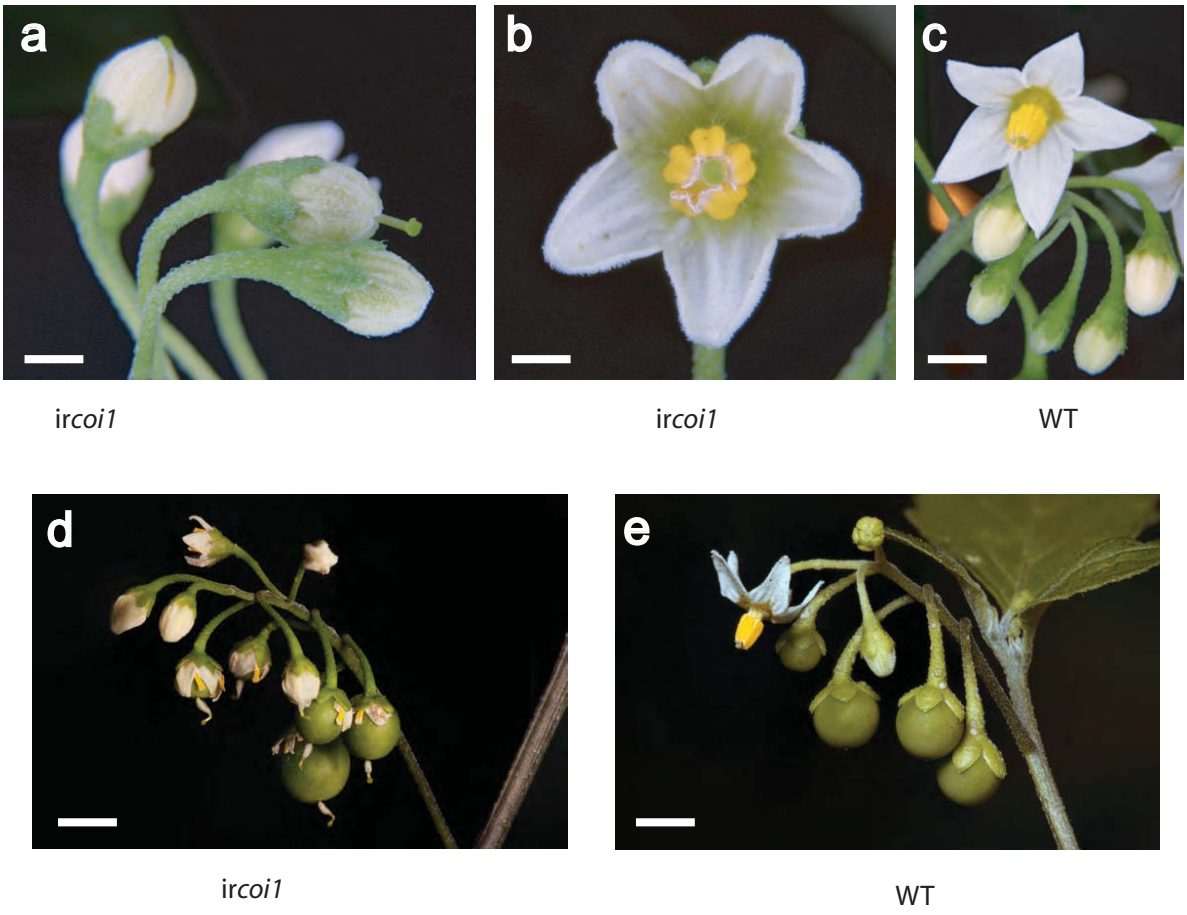

Figure S4. Flower and berry phenotypes of ir*coi1* plants.
(a,b,c) Photographs of buds and flowers of ir*coi1* and WT plants, showing the protruding style of ir*coi1* flowers. (d,e,) Berry development of ir*coi1* and WT plants, showing that the corollas and the styles do not senesce during fruit ripening. Scale bars represent (a) 2.5, (b) 2, (c) 5, (d) 10 and (e) 10 mm.

Chapter 5

JA-Ile Signaling in *Solanum nigrum* is Not Required for Defense Signaling in Nature

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In preparation

JA-Ile signaling in *Solanum nigrum* is not required for defense responses in nature

Abstract

Jasmonate signaling plays a central role in activating the plethora of responses that are elicited by herbivory. *Solanum nigrum* plants silenced in the expression of genes involved in JA biosynthesis (ir*lox3*), conjugation (ir*jar4*) and perception (ir*coi1*) were used to study the function of these genes in the field and in the regulation of transcriptional and metabolic responses. In the field, *Noctuidea* damage was 4 to 5-fold higher on ir*lox3* and ir*coi1* than on WT plants, whereas damage to ir*jar4* plants was similar to WT levels. Damage rates reflected survival rates: fewer ir*lox3* (78%) and ir*coi1* (22%) plants survived compared with ir*jar4* and WT plants of which all plants survived. Gene expression profiling in leaves 3h after simulated herbivory revealed differential regulation of ~700 genes in ir*lox3* and ir*coi1* plants but of only 6 genes in ir*jar4* compared to WT plants. Surprisingly, transcriptional responses were not reflected in metabolomic responses; 48h after simulated herbivory ir*jar4* plants showed a 50% overlap in their metabolic profile with ir*coi1* plants. Together, these results reveal that SnJAR4 does not play a role direct herbivore defense, but suggest that SnJAR4 is involved in other responses to herbivory.

Introduction

Jasmonate signaling is essential in the activation of a myriad of responses against herbivorous insects (Howe and Jander, 2008). These responses include the activation of direct and indirect defense responses (Halitschke and Baldwin, 2003; Thaler *et al.*, 2002), which requires a re-organization of both the primary and secondary metabolisms (Bennett and Wallsgrove, 1994; Schwachtje and Baldwin, 2008). Consequently, plants with reduced jasmonate biosynthesis or perception show decreased resistance against herbivores and pathogens in the glasshouse (Halitschke and Baldwin, 2003; Li *et al.*, 2004; McConn *et al.*, 1997; Vijayan *et al.*, 1998; Xie *et al.*, 1998). Importantly, when these jasmonate-deficient plants are transplanted into their natural environments, not only are these plants more susceptible to existing herbivores, but they also become susceptible to non-host insect species (Kessler *et al.*, 2004; Paschold *et al.*, 2007).

Jasmonic acid (JA) biosynthesis starts with the release of 9,12,15-octadecatrienoic acid (18:3) and 7,10,13-hexadecatrienoic acid (16:3) from membrane lipids in the chloroplast, after which these fatty acids are oxygenated to 13*S*-hydroperoxy-18:3 or -16:3, a step mediated by a 13-lipoxygenase (LOX). Subsequent oxidation and cyclization steps lead to the formation of (9*S*,13*S*)-12-oxo-

phytodeinoic acid (OPDA), which is transported to the peroxisome where several steps of β-oxidation result in the formation of (3*R*,7*S*)-JA [reviewed by Wasternack (2007)]. LOXs are an essential step in JA biosynthesis, plants with reduced LOX expression showed 50-95% reductions in JA levels after wounding in different plant species (Halitschke and Baldwin, 2003, McConn *et al.*, 1997, Chapter 4). JA can be metabolized into a variety of jasmonates (Wasternack, 2007), and the conjugation of JA to isoleucine to form jasmonyl-isoleucine (JA-Ile) has been shown to be important for the activation of defense responses (Staswick and Tiryaki, 2004). This conjugation step is mediated by JASMONATE RESISTANT 1 (JAR1) in Arabidopsis thaliana, by JAR4/6 in Nicotiana attenuata and JAR4 in *Solanum nigrum* (Staswick and Tiryaki, 2004, Wang *et al.*, 2008a, Chapter 4). In *A. thaliana*, JA-Ile is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1) and the transcriptional repressor JASMONATE ZIM DOMAIN1 (JAZ1), thus activating gene transcription (Chini *et al.*, 2007; Sheard *et al.*, 2010; Thines *et al.*, 2007). *A. thaliana jar1-1* plants show decreased resistance to the soil fungus *Pythium irregulare* (Staswick *et al.*, 1998), but do not show the lack of defense responses reported for *coi1-1* plants (Li *et al.*, 2004; Suza and Staswick, 2008; Xie *et al.*, 1998). *N. attenuata* inverted-repeat (ir)*jar4/6* plants show reduced levels of nicotine and proteinase inhibitors, and it was shown that NaJAR4/6 is responsible for the activation of several defense-related genes, but these responses differ from anti-sense (as)*lox3* plants (Wang *et al.*, 2008a). Moreover, transcriptional analysis of *A. thaliana jar1-1* mutant plants show no large changes compared to WT controls (Suza and Staswick, 2008), while in a different study was shown that COI1 controls a large proportion of the wound-activated and repressed genes in *A. thaliana* (Devoto *et al.*, 2005; Wang *et al.*, 2008b). A few hypotheses about the differences between plants deficient in JAR, COI1 and LOX have been proposed. One is that the remaining levels (ca. 10% of WT) of JA-Ile in *A. thaliana jar1-1* and *N. attenuata* ir*jar4/6* plants are sufficient for the activation of defense responses (Suza and Staswick, 2008). The crystal structure of the COI1/JAZ1 complex shows that JA-Ile binds specifically to this complex (Sheard *et al.*, 2010), however, other JAZ genes have been identified, and it remains a possibility that other jasmonates could bind to a COI complex involving alternative JAZ proteins. An important aspect of the defense against insects is the feeding-induced accumulation of toxic or deterring metabolites. Many herbivore-induced traits have been identified, and one of the first discovered induced defenses were protease inhibitors (PIs), which increased substantially in wounded tissue and were later shown to be jasmonate-dependent (Green and Ryan, 1972; Li *et al.*, 2002). Jasmonate signaling has also been shown to play a role in the accumulation of various wound-induced metabolites, for example the accumulation of nicotine has been shown to be a jasmonate-induced defense trait in *N. attenuata* (Halitschke and Baldwin, 2003; Steppuhn *et al.*, 2004), while

anthocyanin production in *A. thaliana* is also a COI1-dependent trait. In *Solanum lycopersicum*, Pearce *et al.* (1998) showed that the phenolic compound, feruloyltyramine, is wound-induced, but its accumulation was COI1-independent.

Advances in mass spectrometry have enabled metabolomics approaches to be used characterize the herbivory induced changes in plants. Jansen (2009) identified compounds from both the plant and the midgut of the feeding insect in the interaction between *Brassica oleracea* and *Pieris rapae*, whereas in the interaction between *Barbarea vulgaris* and *Phyllotreta nemorum*, Kuzina *et al.* (2009) showed that saponins and other compounds correlated strongly to the survival of *P. nemorum* on an F2 population of susceptible and resistant *B. vulgaris* parents. Moreover, the wound response in *A. thaliana* has been analyzed using UPLC-TOF analysis, resulting in the discovery of several novel jasmonates (Glauser *et al.*, 2010; Glauser *et al.*, 2008). Recently, the metabolic responses of *N. attenuata* after perception of *Manduca sexta* herbivory was analyzed by LC-QTOF, and this analysis revealed that diterpene glycosides and spermidines were strongly induced by herbivory (Gaquerel *et al.*, 2010).

S. nigrum has been established as a model system to understand defense responses in a wild species. In a previous study, stably silenced lines for JA biosynthesis (ir*lox3*), conjugation to JA-Ile (ir*jar4*) and reception (ir*coi1*) were reported (Chapter 4). In that study it was shown that in *S. nigrum*, local JA-Ile signaling is not essential for activation of the systemic defense response in the glasshouse. Here, we ask what role JA-Ile-dependent signaling plays in *S. nigrum*'s defense responses, and study gene transcription and metabolic responses to herbivory in ir*jar4*, ir*lox3* and ir*coi1* plants. Field experiments with transgenic plants were used to identify the role of JA-Ile in plant defense in a natural environment.

Material & Methods

Glasshouse treatments and conditions

Solanum nigrum L. inbred line Sn30 (Schmidt *et al.*, 2004) were used in all experiments, and were germinated and cultivated as described in (Schmidt and Baldwin, 2006). All experiments were performed with approximately 4 week-old plants that just started flowering.

W+OS elicitations were performed by rolling with a fabric pattern wheel three times at each side of the midvein, and immediately applying 20µL 1:5 diluted *M. sexta* oral secretions and regurgitants (OS) to the resulting puncture wounds. OS was collected from 3rd to 4th instar *M. sexta* caterpillars that fed on WT plants. After treatment, samples were harvested by harvesting whole leaves and flash freezing these in liquid nitrogen.

Field experiments

Plants were grown in an irrigated field plot in Santa Clara, UT, germination was induced as described in (Schmidt and Baldwin, 2006), and seeds were transferred to jiffy pots. Three-week old plants were transferred to the field, and irrigated every 2-3 days. The field was divided in 50 1 × 1 m squares , each containing a ir*lox3*, ir*jar4*, ir*coi1* and WT plant, in alternating positions. Around the edge of the field plot ~100 WT plants were planted to attract native insects, and buffer any edge effects on the experimental plants. Herbivore screens were performed at 3, 5 and 9 days after transplantation by estimating the herbivore damage on each plant, scored as a percentage of the total plant leaf area. Jasmonate kinetics were performed by eliciting leaves with W+OS or W+W (same procedure as W+OS, replacing *M. sexta* OS with ddH_2O containing 0.02% (v/v) Tween-20), leaf samples were harvested after different times, immediately frozen on dry ice and kept at -20°C until analysis. For PI analysis, plants were elicited by W+OS induced on two consecutive days, and samples harvested three days after the first elicitation. JA and JA-Ile analysis was performed as described previously (Chapter 4), briefly, samples were extracted with ethyl acetate containing a labeled internal standard, evaporated and reconstituted in 70% (v/v) MeOH. Extracts were analyzed by LC-MS/MS in MRM mode. Proteinase inhibitor analysis was performed using the radial diffusion assay described by Jongsma *et al.* (1994).

Microarray experiments

For microarray analysis, plants were treated by W+OS and tissue harvested after 3h. Three biological replicates were used, each consisting of five pooled leaves from individual plants. RNA was isolated according to Schmidt and Baldwin (2009), and mRNA isolation, cDNA synthesis, labeling and hybridizations were performed as described by TIGR with the protocol described at: http://jcvi.org/potato/sol_ma_protocols.shtml. WT cDNA was labeled with Cy5, and cDNA from the transgenic lines with Cy3. TIGR potato 10Kv4 chips were used for all hybridizations, detailed information about these chips can be found under http://jcvi.org/potato/sol_ma_microarrays.shtml. Microarray data was quantified using AIDA software, and normalized using LOWESS. SAM (statistical analysis for microarrays) (Tusher *et al.*, 2001) was used for false discovery rate (at the 5% level) and significance analysis. A fold-change cut-off of >1.5 <0.67 was applied.

Metabolic analysis

For analysis of the metabolome, ir*jar4*, ir*coi1* and WT plants were elicited by W+OS, and leaf samples harvested at 0 (uninduced control), 12, 24 and 48h after elicitation. Leaf samples were ground to a fine powder in liquid nitrogen and 50 mg tissue was extracted with 1 mL MeOH containing 1% (v/v) formic acid. Samples were sonicated for 20 minutes and subsequently centrifuged for 20 min. Extracts were filtered through a 0.22 µm membrane to remove particles. Five µL sample were injected into a ultraperformance liquid chromatography-quadrapole time of flight (UPLC-QTOF) instrument (Waters HDMS Synapt). Mobile phases were (A) H_2O containing 5/0.1% (v/v) acetonitrile/formic acid (FA) and (B) acetonitrile containing 0.1% FA. The following gradient was used: 0-17.3 min, linear gradient from 0-22% B (v/v), 17.3-27.3 min 22-28% B, 27.3-28 min 28-40% B, 28-28.5 min 40-100% B, 28.5-30 min 100% B, 30-30.5 min 100-0% B, 30.5-32 min 0% B, using a 100 × 2.1 mm, 1.7 µm UPLC BEH column C18 column (Waters Acquity) with a flow of 0.3 mL min^{-1}. The following settings were applied during the LC-MS runs: capillary voltage at 3.0 kV; cone voltage at 28 eV; collision energy at 4 eV. For the LC-MS/MS runs collision energies were set to 10 - 25 eV. The MassLynx software version 4.1 (Waters Inc.) was used to control the instrument and calculate accurate masses. For quality control, samples were injected in a randomized order, and a standard mix containing 15 standards was injected every ten samples. Metabolomics data was extracted using XCMS under R (Smith *et al.*, 2006), using the script supplied as an .R file in the supplemental materials. Because in-source fragmentation and natural occurring isotope ratios lead to many detected ions that belong to the same compound, a method was developed to cluster these. Based on the rationale that ions belonging to the same compound will show a high correlation to each other (between replicates and the different time points) and will have the same retention time, ions with both a Pearson correlation of >0.9 and an average retention time of <5s were added to a single cluster. The basis for this method has been described and validated by Gaquerel *et al.* (2010). Data was normalized by the total peak area, and subsequently *log*-2 transformed. For statistical analysis, the R-based EDGE software (Storey *et al.*, 2005) was used. For multiple analyses in EDGE, such as performed for the clusters that were induced over time, the same seed (8888) was used.

Results

Jasmonate signaling in the field

To test the role of jasmonate production, conversion and perception on herbivore resistance and plant survival, ir*lox3*, ir*jar4* and ir*coi1* plants were grown along with a WT control in an irrigated field plot at the Lytle Ranch Preserve, Santa Clara, Utah, USA.

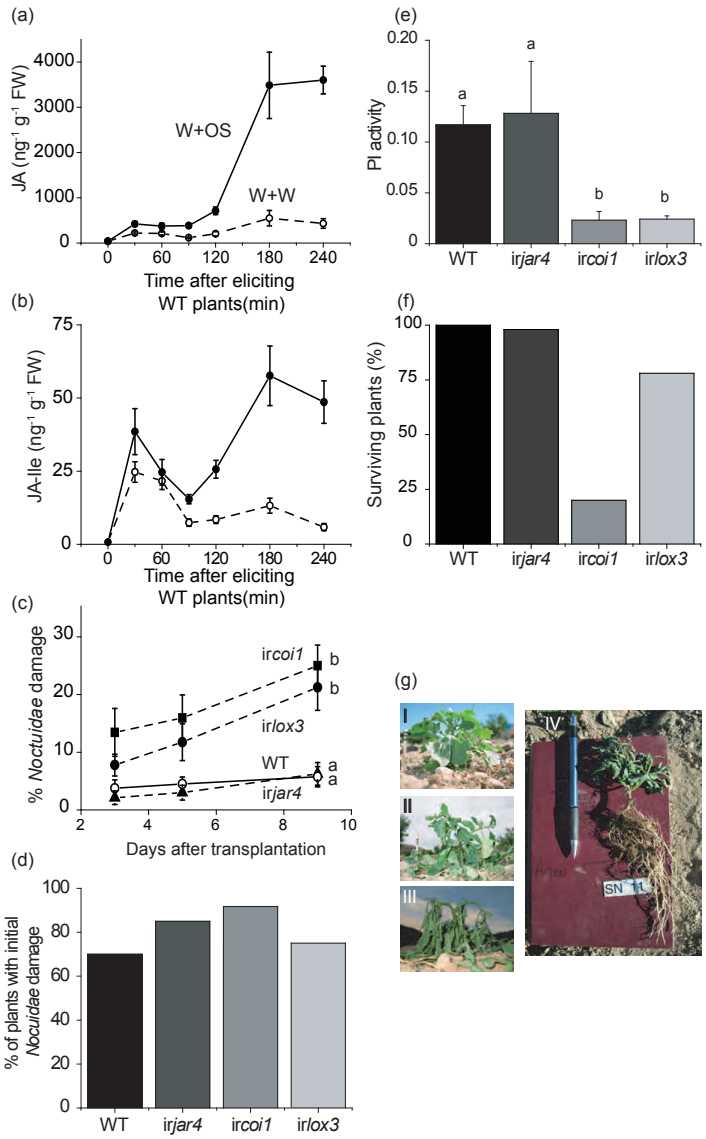


Figure 1. Jasmonate signaling in nature. (a,b) Jasmonic acid (JA) and jasmonyl-isoleucine (JA-Ile) accumulation in field-grown WT plants. Plants were wounded by rolling a pattern wheel three times on each side of the midvein, and either *M. sexta* oral secretions and regurgitants (W+OS, solid lines, closed circles) or H_2O (W+W, dashed lines, open circles) were immediately applied to the resulting puncture wounds. Leaves were harvested on dry ice after different time points and analyzed for JA. Means of five biological replicates are presented, error bars represent ± S.E. (c) *Noctuidea* damage on WT (solid line, open circles), ir*lox3* (dashed line, closed circles), ir*jar4* (dashed line, triangles) and ir*coi1* (dashed line, squares) plants. Damage was estimated on three different days, different letters indicate a significant difference (univariate ANOVA $F_{3,67}$=10 followed by a Scheffé *post-hoc* test, P<0.05). Means of 11 to 20 biological replicates are presented, error bars represent ± S.E. (d) Initial *Noctuidea* damage on WT, ir*lox3*, ir*jar4* and ir*coi1* plants. Initial damage was scored as presence/absence 8 days after transplantation into the field plot, based on wrinkled young leaves and the presence of silk on these leaves (See Fig S1). (A binominal test comparing WT to ir*jar4*, ir*lox3* and ir*coi1* showed no significant difference, P>0.05, n=11 to 20). (e) Proteinase inhibitor levels of ir*lox3*, ir*jar4* and ir*coi1* plants. Leaves were elicited on two consecutive days by W+OS treatment and plants harvested three days after the first elicitation and proteinase inhibitor activity analyzed. Different letters indicate a significant difference (univariate ANOVA $F_{3,33}$=12.9 followed by a Scheffé *post-hoc* test, P<0.05). Means of 8 to 10 biological replicates are presented; error bars represent ± S.E. (f) Survival of WT, ir*jar4*, ir*coi1* and ir*lox3* plants in the field. One month after transplanting plants the number of surviving plants was scored, and presented as a percentage. Starting n was 50. (g) Photographs of ir*coi1* plants suffering from complete vascular collapse. Symptom-free ir*coi1* plant (I), ir*coi1* plants with starting symptoms (II), ir*coi1* plant with severe symptoms (III), excavated ir*coi1* plant showing no obvious disease signs (IV).

First, JA and JA-Ile accumulation was analyzed after W+W and W+OS treatment in field-grown WT plants, and in Fig 1a and b it is shown that the JA and JA-Ile burst show a wound-induced accumulation, and a late, OS-induced second burst. This is very similar to what is shown for JA and JA-Ile dynamics of glasshouse-grown *S. nigrum* plants (Chapter 4).

Analysis of herbivore damage on field-grown plants showed that *Noctuidae* damage, scored by recording the percentage *Noctuidae* damage on the whole plant, was 20 and 25% for ir*lox3* and ir*coi1* plants, respectively, while ir*jar4* plants suffered little damage: ~5% damage

was detected on these plants, levels similar to WT plants (Fig 1c). These differences however, could have originated from oviposition preferences, introducing a bias. Because *Noctuidae* eggs are barely visible with the human eye, initial *Noctuidae* feeding damage (characterized by wrinkled, young leaves and the presence of silk, see Fig. S1) was scored as an indirect measure of oviposition preferences. Fig 3d shows that no significant differences in initial herbivore damage between the genotypes could be observed.

Proteinase inhibitor (PI) activity is a trait involved in *S. nigrum*'s defence against herbivores (Hartl *et al.*, 2010), PI activity was analyzed after leaves were elicited twice by W+OS treatments, and this tissue was analyzed three days after the first for elicitation. The results showed that ir*lox3* and ir*coi1* plants had reduced PI levels, both to 20% of WT, while ir*jar4* plants PI levels did not differ from WT (Fig 1e).

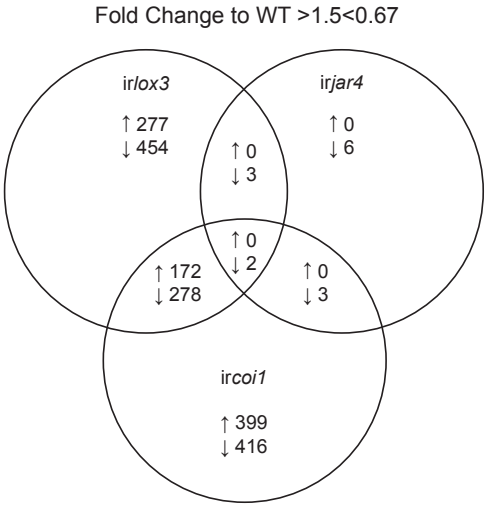
One month after transplanting the plants into the field, plant mortality was scored; all WT plants and 98% of the ir*jar4* plants survived, while 78% and 20% of the ir*lox3* and ir*coi1* plants survived, respectively (Fig 1f). ir*coi1* mortality was not only caused by herbivory, but seven healthy-looking plants suffered from spontaneous, complete wilting; the first symptoms were observed in the morning, and the plants died within 24 h. However, no indication of a leaf or root pathogen could be observed (Fig 1g).

Transcriptional profiling in jasmonate-deficient plants

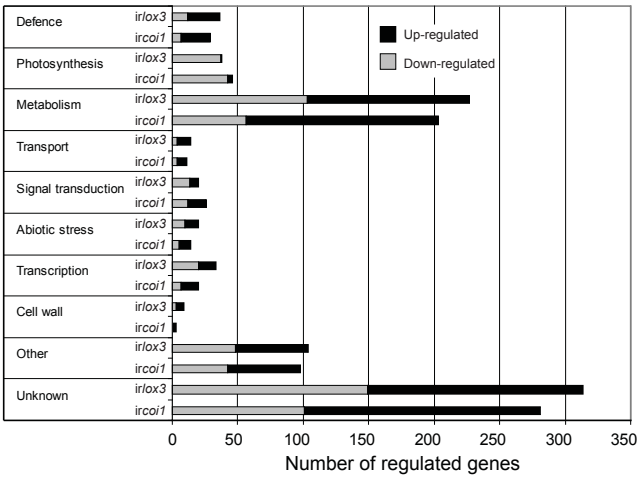
To assess the short-term transcriptional impact of deficiencies in jasmonate perception, production and conjugation, transcriptional profiling on ir*lox3*, ir*jar4* and ir*coi1* plants was performed. The TIGR potato 10k v4 microarray was used in previous studies with *S. nigrum*, and revealed that *S. nigrum* responds strongly to *M. sexta* feeding and MeJA treatment (Schmidt *et al.*, 2005). Previous work on jasmonate signaling in *S. nigrum* showed that JA levels reach their maximum at 3h after W+OS, but that the main JA-Ile burst occurs at 30 min after elicitation, whereas between 90 min and 4h a jasmonate-dependent systemic signal leaves the local leaf (Chapter 4). Thus, we chose to use tissue from W+OS treated leaves, harvested 3h after W+OS elicitation. Microarrays experiments were performed by hybridizing cDNA from WT against each of the inverted-repeat lines. Microarrays were scanned and quantified using AIDA software, after which they were normalized according to standard protocols (see Material and Methods). The results showed that SnLOX3 and SnCOI1 had a strong effect on gene expression at 3h after W+OS elicitation: 277 and 399 genes were up-regulated compared to WT in both genotypes, respectively, while 454 and 416 genes were down-regulated (Fig 2a). Between the genotypes, both in up- and down-regulated genes, there was approximately 50% overlap in gene identity (Fig 2a). Most of these commonly regulated

genes were related to metabolism, which were both up- and down- regulated. For example, genes related to photosynthesis, which were mostly upregulated in ir*lox3* and ir*coi1*, and defense-related genes were mostly down-regulated in ir*lox3* and ir*coi1* plants (Fig 2b). In ir*jar4* plants however, only 6 genes changed their W+OS elicited expression compared to WT, none up and 6 down, of which 4 and 2 responded similarly in ir*lox3* and ir*coi1*, respectively. The genes regulated in ir*jar4* plants were not defense-regulated, but involved in other processes (Fig 2c).

(a)



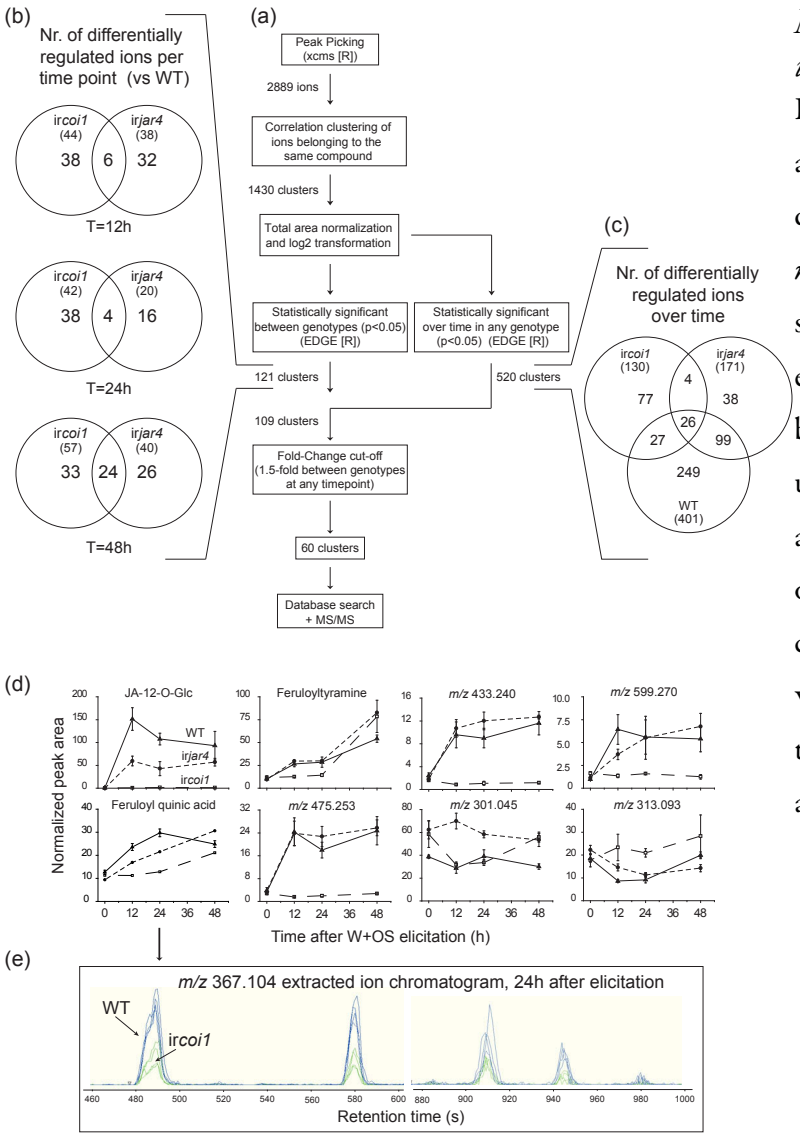
(b)



(c)

Genes regulated in ir*jar4* plants	FC	q-value	Regulated in ir*lox3*	Regulated in ir*coi1*
Chaperone protein DnaJ (*A. thaliana*)	0.43	0	x	
Auxin response factor 6 (*A. thaliana*)	0.65	0		x
Extracellular dermal glycoprotein (*A. thaliana*)	0.65	2.92		
Protein phosphatase C (*A. thaliana*)	0.65	0	x	x
Homeobox-leucine zipper protein ATHB-12 (*A. thaliana*)	0.65	3.86	x	x
Late embryogenesis abundant protein Lea5 (*Citrus sinensis*)	0.66	0		

Figure 2. Transcriptional profiling of ir*lox3*, ir*jar4* and ir*coi1*, compared to WT plants. WT, ir*lox3*, ir*jar4* and ir*coi1* plants were elicited by W+OS and leaf tissues harvested after 3h. RNA was extracted, and labeled CDNA synthesized. Labeled WT CDNA was hybridized against ir*lox3*, ir*jar4* and ir*coi1* CDNA samples on TIGR potato 10k v4 microarrays, and statistical analysis performed as described in the Material and Methods section. (a) Venn diagram comparing the transcripts of ir*lox3*, ir*jar4* and ir*coi1* to those of WT plants. Significantly regulated genes with a fold-change >1.5 and <0.67 are showed. (b) Genes regulated in ir*lox3* and ir*coi1* plants from different functional categories. Total bar length is the total number of regulated genes, up-regulated genes are displayed in black, down-regulated genes in grey. (c) All six genes regulated by in ir*jar4* plants, FC is fold-change.



Metabolic profiling of WT, irjar4 and ircoi1 plants

Plant secondary metabolites play an important role in herbivore defense, and we analyzed *S. nigrum*'s metabolome after simulated herbivory. For this experiment, a UPLC-QTOF-MS based metabolomic approach was used and negative ions were analyzed. The metabolic responses of WT, ir*jar4* and ir*coi1* plants were compared by eliciting leaves with W+OS and harvesting the tissue at t = 0 (unelicited control), 12, 24 and 48h.

Figure 3. Metabolic analysis of ir*jar4*, ir*coi1* and WT plants. Plants were W+OS elicited, leaf material harvested after different times, extracted and analyzed by UPLC-QTOF-MS. (a) Flow diagram used for metabolic analysis. Peaks were picked using XCMS and ions with a Pearson correlation of >0.9 and a retention time difference of <5s were clustered and subsequently normalized and *log-2* transformed. Statistical analysis was performed using EDGE as described in Storey *et al*., (2005), both to identify clusters significantly regulated between genotypes and clusters that were significantly regulated over time. To identify clusters important for herbivore resistance, both EDGE analyses were combined, and a >1.5 <0.67-fold cut-off filter was applied. With the ions, database searches and MS/MS experiments were performed. (b) Venn diagrams comparing ir*jar4* and ir*coi1* plants at individual time points. Clusters of ir*jar4* and ir*coi1* plants were compared to WT plants, and significant clusters are displayed per time point (Student's *t*-test after Bonferroni correction, *P*<0.025), numbers in brackets are the total number of clusters regulated in the genotype. (c) Venn diagrams comparing ion clusters significantly regulated over time in WT, ir*jar4* and ir*coi1* plants. Numbers in brackets are the total number of clusters regulated in the genotype. (d) Eight selected regulated ions, showing different induction patterns. Three compounds, 12-*O*-glc-JA, feruloyl quinic acid and feruloyltyramine were putatively identified based on their mass spectra. For the unknown compounds, the most likely deprotonated molecular ions are presented as *m/z*. All points represent the means of 3 to 4 biological replicates, error bars represent ± S.E. (e) Extracted ion chromatogram of the *m/z* corresponding to 5 different isoforms of feruloyl quinic acid, all with the same fragments in extracted ion chromatograms with a *m/z* of 367.104 in WT (black chromatograms) and ir*coi1* (green chromatograms) plants with 4 biological replicates of each genotype.

Based on the available literature on *S. nigrum*, we expected to detect glycoalkaloids such as solasodine and solamargine [e.g. Eltayeb *et al.*, (1997)], however, these were not be detected in *S. nigrum* leaf extracts. However in green berries extracted with the same procedure, these compounds were abundantly present (data not shown). For metabolomic analysis, chromatograms were aligned, ions extracted and integrated using XCMS (Smith *et al.*, 2006), resulting in 2889 detected ions. Correlation-based clustering as described in Material and Methods reduced the number of ions from 2889 ions to 1430 clusters (Fig 3a). A statistical package optimized for the analysis of large-scale datasets over time, EDGE (Storey *et al.*, 2005), was used to identify clusters that differed among genotypes at particular times. This revealed that 121 clusters were significantly regulated among the genotypes. However, EDGE only calculates the probability of a difference among the genotypes, but no post-hoc test is available to calculate between which pair of genotypes, or at what time these differences occur. To resolve these important details, an additional analysis was performed on these 121 clusters by comparing ir*jar4* and ir*coi1* plants to WT at individual time points. This analysis revealed that after 12 and 24h after elicitation, in both ir*coi1* and ir*jar4* plants, 20 to 44 ion clusters are regulated, but with little overlap (4 and 6 clusters, respectively). However, after 48h ir*coi1* and ir*jar4* regulated the largest number of clusters (57 and 40, respectively) which had a ~50% overlap which each other (Fig 3b).

Parallel to the above analysis, EDGE was used to identify clusters that showed a regulated pattern over time, resulting in 520 significantly regulated ion clusters. Of these, 401 were regulated in WT, 171 in ir*jar4* and 130 clusters in ir*coi1* plants. A comparison revealed a 20% overlap between ir*coi1* and ir*jar4* plants, and of the 401 clusters regulated in WT plants, only 26, or 6.5%, were regulated independently from SNJAR4 and SNCO11 (Fig 3c).

To find substances that could play roles in defense, ions clusters that were both regulated between the genotypes and were elicited after W+OS elicitation were combined, resulting in 109 clusters. Subsequently, these were filtered by a fold-change cut-off of >1.5 <0.67 -fold between any genotype, at any time point, leaving 60 clusters. These 60 clusters were entered in a database search, and some were selected for MS/MS experiments. Finally, JA-12-*O*-glucose (JA-12-*O*-glc), feruloyl tyramine and feruloyl quinic acid were tentatively identified (Fig S2 for MS/MS spectra), while other, unknown ion clusters showed various accumulation patterns (Fig 3d). Because there are different isoforms of feruloyl quinic acid, all with the same fragments (Clifford *et al.*, 2006), an extracted ion chromatograms with a m/z of 367.104 showed that at least 4 isoforms are present and regulated in SNCO11 plants (Fig 2e).

Discussion

Short-term gene regulation is SnJAR4 independent

Transcriptional profiling revealed that when ir*jar4* plants were compared to WT, six genes were down-regulated in ir*jar4* plants (Fig 2a,c). Of these genes, the auxin response factor 6 is involved in jasmonate biosynthesis in flowers (Nagpal *et al.*, 2005). Moreover, the chaperone protein DnaJ, the extracellular dermal glycoprotein, the protein phosphatase C, the homeobox-leucine zipper protein HB12 and the late embryogenesis abundant protein (lea5), are all involved in responses to drought or salt stress in other species (Du *et al.*, 2008; Jiang *et al.*, 2007; Naot *et al.*, 1995; Olsson *et al.*, 2004; Sheen, 1998). The induction of a *N. attenuata* homologue of At*HB12* (Na*HD20*) was also shown to be down-regulated after wounding in ir*jar4/6*, ir*lox3* and ir*coi1* plants (Ré *et al.*, 2010). Moreover, Wang *et al.* (2008a) showed that in *N. attenuata* ir*jar4/6* plants, genes coding for direct defenses such as PIs and polyphenol oxidases were impaired compared to WT, whereas transcriptional studies with *A. thaliana* jar1 plants showed that gene expression of several defense-related genes such as VSP2 and PDF1.2 was not different from WT plants (Suza and Staswick, 2008). Because drought stress-related and not direct defense genes (which were represented on the microarray) were deregulated in ir*jar4* plants, which suggested that SnJAR4 plays a role in abiotic stress responses following herbivory, but not in the direct defense response.

In contrast to ir*jar4* plants, regulation of most defense-related genes was strongly reduced in ir*coi1* and ir*lox3* plants (Fig 2b). Defense-related genes have been found to be commonly regulated by COI1 and LOX in different plant species; Devoto *et al.* (2005) described that direct defense-related genes were de regulated in *A. thaliana coi1-1* plants, while transcriptional analysis on *N. attenuata* as*lox3* plants also showed the down-regulation of several defense-related genes compared to WT plants (Halitschke and Baldwin, 2003). The increased transcription of photosynthesis-related genes in ir*lox3* and ir*coi1* indicates that the herbivore-induced reduction of photosynthesis as reported by Zangerl *et al.* (2002) is a jasmonate-dependent process, as reported previously (Zavala and Baldwin, 2006). Circa 200 SnCOI1- and SnLOX3-regulated genes were related to metabolism, which is consistent with studies showing that herbivory induces large changes in the primary and secondary metabolism of different plant species (Schwachtje and Baldwin, 2008; Zhang and Memelink, 2009). The 50% transcriptional overlap between ir*lox3* and ir*coi1* plants shows that there are JA-dependent, SnCOI1-independent, and SnCOI1-dependent, JA-independent signaling involved in this regulation. For example, transcriptional profiling in *A. thaliana* also showed COI1-independent gene regulation (Reymond *et al.*, 2000). Signaling through JA-independent mechanisms was previously shown to occur through OPDA-dependent signaling (Stintzi *et al.*, 2001).

SnJAR4 and SnCOI1 regulate most herbivore-induced metabolites

To investigate the impact of jasmonate signaling on herbivoreSnCOI1-induced metabolomic rearrangements, ir*jar4*, ir*coi1* and WT plants were elicited by W+OS, tissue harvested after different times and analyzed by UPLC-QTOF-MS. Before statistical analysis, ions likely belonging to the same compound were clustered based on their correlation and retention time, resulting in a total of 1430 clusters (Fig 3a). An analysis of all the clusters that changed over time showed that 94% of the WT-induced clusters were SnJAR4 or SnCOI1 dependent (Fig 3c), indicating that jasmonate signaling-dependent mechanisms are mainly responsible for the induced changes in *S. nigrum*'s metabolome. Contrary to its minor role in inducing short-term gene regulation, ir*jar4* plants had a different metabolic profile from WT plants, and showed a 20% overlap with the SnCOI1-regulated clusters (Fig 3c). An analysis comparing ir*jar4* and ir*coi1* plants with WT plants at individual time points showed that SnJAR4 regulated 20 to 40 metabolites, and that the overlap with SnCOI1-regulated metabolites was ca. 50% after 48h (Fig 3b), indicating that there are COI1-dependent, JA-Ile-independent metabolic responses to herbivory, particularly at the earlier time points after elicitation. Previous work on *S. nigrum* demonstrated that the short-term activation (4h) of systemic defenses was strongly inhibited in ir*coi1*, but was not significantly affected in ir*jar4* plants (Chapter 4), indicating that signaling between JA-Ile and SnCOI1 is indeed weak at earlier time points.

Identification of some of the metabolites showed that our *S. nigrum* accession [Sn30, as described by Schmidt *et al.* (2004)] did not contain detectable levels of glycoalkaloids in its leaves. This is in contrast to what has been published previously (Eltayeb *et al.*, 1997). Instead, saponins were identified as major compounds, which are known compounds in *S. nigrum* (Zhou *et al.*, 2006). In green, unripe berries however, glycoalkaloids were abundantly present, which might indicate that there is large temporal variation in leaf-accumulated glycoalkaloids among different *S. nigrum* genotypes

The identification of JA-12-O-glc shows that JA accumulation continued for 48h after OS elicitation, while previous results showed that in *S. nigrum*, local jasmonates accumulated for a few hours, not days (Chapter 4). Because previous experiments established that jasmonate accumulation up to only 4h is important in the activation of systemic signaling, is the role of the late accumulation of JA-12-*O*-glc unclear. One hypothesis is that this jasmonate is used as a pool to quickly release JA upon multiple wounding events. The accumulation of JA-12-*O*-glc was reduced to 50% in ir*jar4* and reduced to 2% in ir*coi1*, compared to WT plants. This is similar to the reductions of 12-OH-JA in ir*jar4* and ir*coi1* plants, with ir*jar4* plants showing less reduction of this hydroxylated jasmonate than ir*coi1* plants at 3h after W+OS elicitation (Chapter 4).

The other identified compounds were the ferulic-acid esters feruloyl quinic acid and feruloyltyramine. Up to five different isoforms of feruloyl quinic acid were detected and regulated in ir*coi1* plants (Fig 3e), while ir*jar4* plants also showed regulation of this compound. Feruloyl quinic acid and its precursors, ferulic and quinic acid, have been implicated as compounds in the defense against herbivores (Leiss *et al.*, 2009; Pearce *et al.*, 1998). Feruloyl tyramine has been implicated in the anti-herbivore response in *S. lycopersicum*, Pearce *et al.* (1998) identified this compound as being 10-fold up-regulated after wounding in *S. lycopersicum*, but its accumulation was not changed in *S. lycopersicum jai-1* plants, mutated in the COI1 gene. Here, we showed that feruloyltyramine is regulated by SnCOI1, but only at 12 and 24h and not at 48h (Fig 3d). This indicates that SnCOI1-dependent signaling is important in the early accumulation of feruloyl tyramine, but that the accumulation at 48h is activated in an SnCOI1-independent manner. Pearce *et al.* (1998) were not able to detect feruloyl tyramine in both wounded and unwounded *S. nigrum* leaves, which is probably due to differences of sensitivity in the detection methods.

SnJAR4 is not required for defense signaling in nature

Field experiments with *S. nigrum* in the Great Basin Desert of the SW USA were performed to assess the impact of jasmonate signaling on plant defense in nature. Although *S. nigrum* is not native to the USA, it is an invasive species there, and occurs throughout the United States.

Herbivory screening showed that *Noctuidae* damage was 4 to 5-fold higher on ir*lox3* and ir*coi1* then on WT plants. Initial *Noctuidea* damage was equally high on all genotypes, indicating that oviposition differences did not significantly contribute to this phenotype. Proteinase inhibitors (PIs) have been shown to play a role in *S. nigrum*'s defense against *Noctuidae* in a natural environment, (Hartl *et al.*, in press), and PI levels in field-grown plants after W+OS induction showed indeed that ir*lox3* and ir*coi1* plants have strongly reduced PI activity, while ir*jar4* plants were not different from WT. These results showed that SnLOX3 and SnCOI1 play an important role in defense signaling in nature, while SnJAR4 does not. This matched the transcriptional profiling, which also showed that most defense-related genes were down-regulated in ir*lox3* and ir*coi1*, but not in ir*jar4* plants. These results are also congruent to other field studies using *N. attenuata* plants silenced for LOX3 and COI1, which showed decreased resistance to herbivores (Kessler *et al.*, 2004; Paschold *et al.*, 2007). Plants lacking JAR expression have, to our knowledge, not been tested in the field so far, but glasshouse experiments with *N. attenuata* ir*jar4/6* plants showed that these have reduced levels of induced PIs, and that *M. sexta* performance is better on ir*jar4/6* then WT plants (Wang *et al.*, 2008a). This indicates that JA-Ile signaling in *N. attenuata* plays a different role in inducing direct defenses than in *S. nigrum*. It has previously been argued that the 10-15% remaining levels of JA-Ile in JAR

silenced or mutant plants is responsible for the induction of defenses (Suza and Staswick, 2008), however, we previously showed that ir*lox3* plants accumulate more JA-Ile than ir*jar4* plants after wounding (Chapter 4), indicating that remaining levels of JA-Ile in ir*jar4* plants are unlikely to explain the 4-fold difference in herbivore damage between ir*lox3* and ir*jar4* plants.

The relatively large metabolic reorganization in ir*jar4* plants had no significant effects on herbivory in the field, indicating that the overlap in metabolites both regulated in ir*coi1* and ir*jar4* plants are not involved in the direct defence against herbivores, but possible against other herbivore-induced responses reported in *S. nigrum*, such as tolerance (Schmidt and Baldwin, 2009). The biphasic OS-elicited JA and JA-Ile accumulation patterns were very similar to what described from glasshouse experiments (Chapter 4) was also observed in the field; Diezel *et al.* (2009) described that also for *N. attenuata*, the JA burst, which is different from *S. nigrum*, is similar in the field and the glass house. This shows that although the kinetic of the jasmonate burst is highly variable between different plant species, this burst is extremely robust to environmental influences. These differences in jasmonate accumulation and its consequences between plant species show that jasmonate accumulation and signaling is a highly specific process, and most likely originates from the millions of years of co-evolutions with specialized herbivores.

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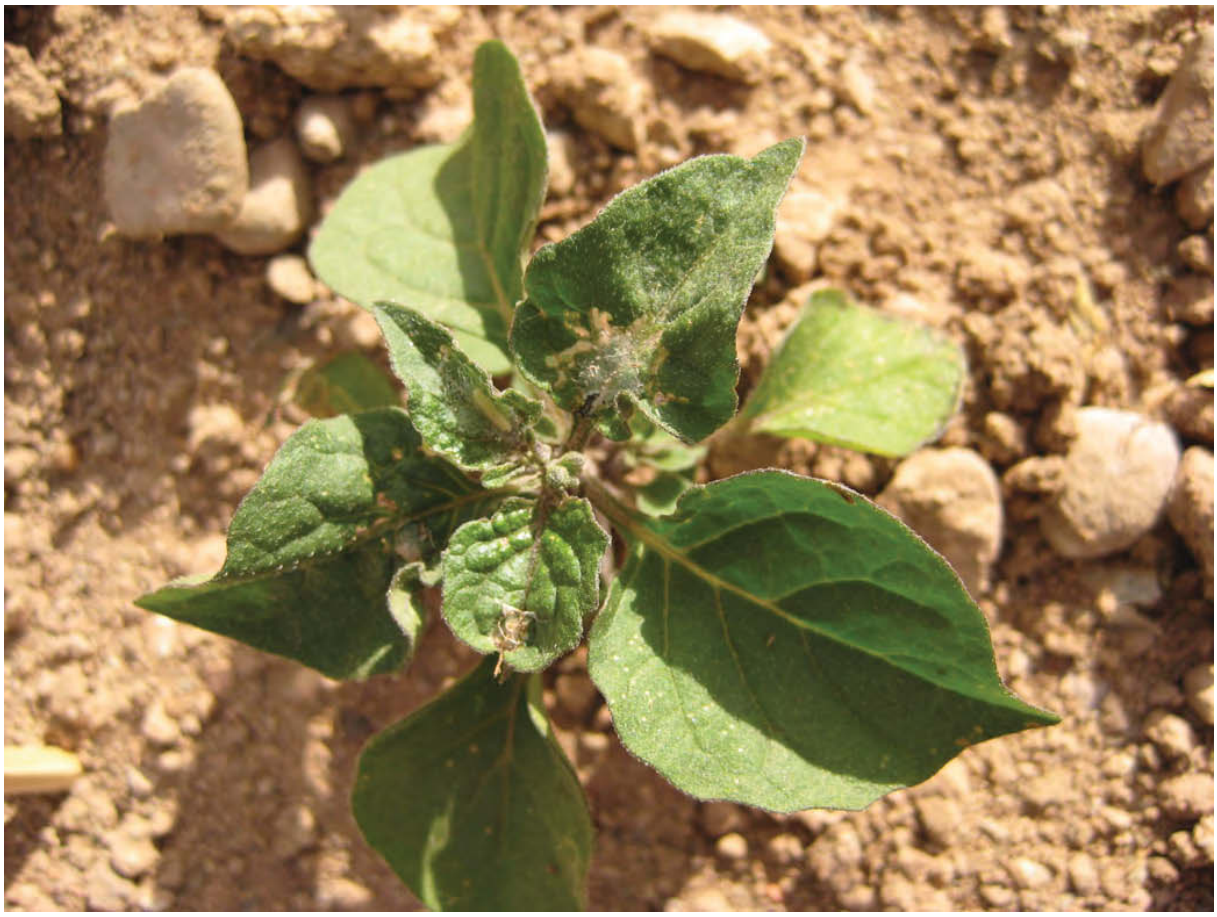


Figure S1, Photograph of initial *Noctuidea* damage on a *Solanum nigrum* plant.
Wrinkled, young leaves and the presence of silk were used as indicators for initial *Noctuidea* damage.

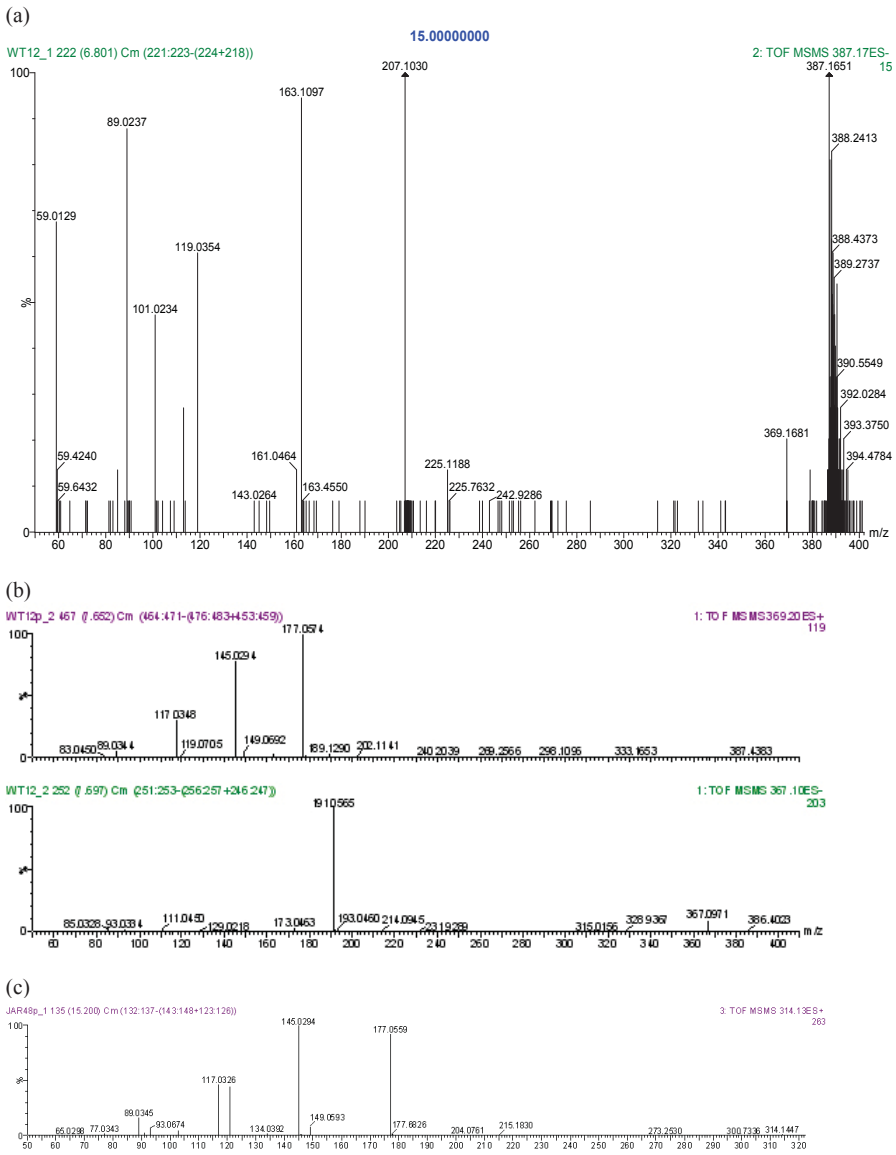
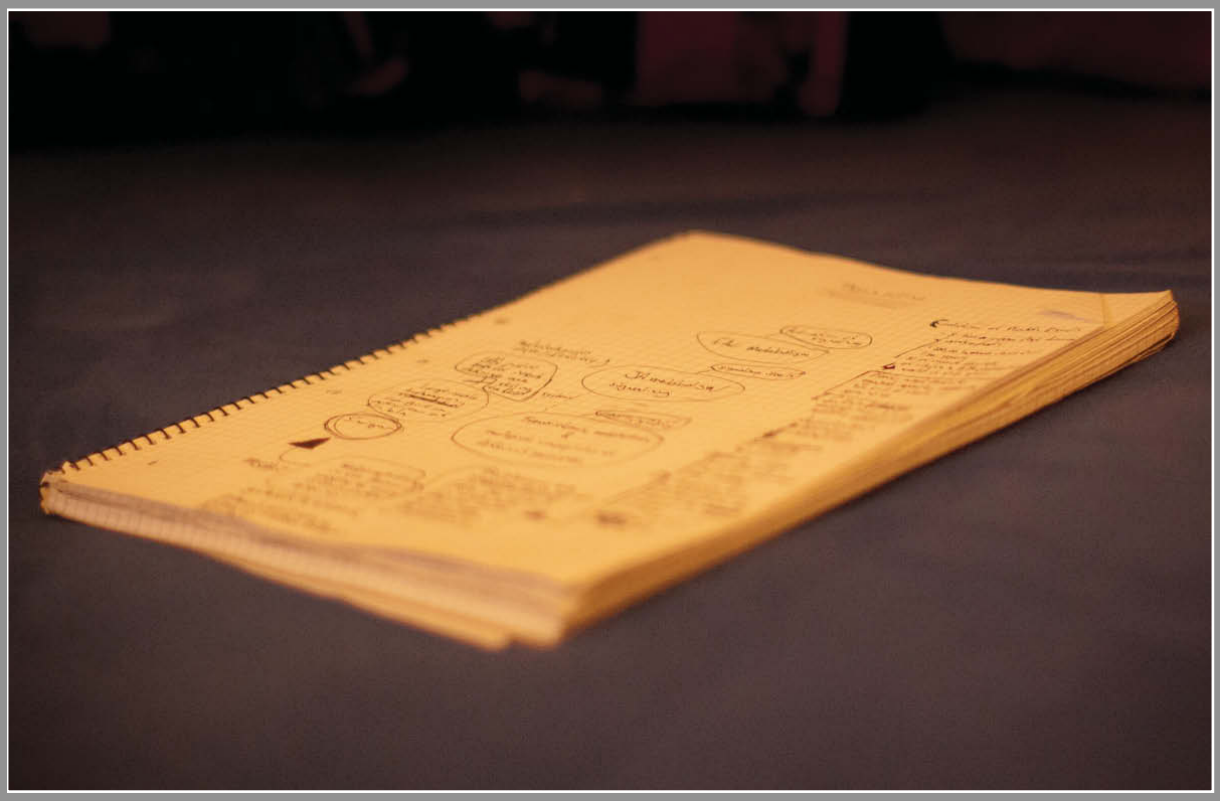


Figure S2 MS/MS Spectra of putatively identified compounds.
For MS/MS experiments, 5 µL of sample were injected into the instrument using the same gradient as described in the Materials and Methods section. Collision energy of 15 eV were applied in the negative mode and 10 eV– in the positive mode. (a) Spectrum of 12-O-glc-JA. Parent ion used for collision was *m/z* 387.165 (negative mode); fragments used for identification: *m/z* 369.1681 (M-H-H_2O), 207.1050 (M-H-H_2O-Hex), 163.1097 (M-H-H_2O-Hex-COO), 59.01 (CH_2COOH-) (b) Spectra of feruloyl quinic acid. Top two panes are MS/MS spectra in the positive mode, lowed two in negative mode. Parent ion used for collision was 367.10 (negative) and 369.20 (positive). Fragments used for identification: Postive mode - *m/z* 177.0574 (M+H-quinic acid), 145. 0294 (M+H-Quin-OCH_3); negative mode - *m/z* 191.0565 (M-H-ferulic acid, resulting in [quinic acid-H]) 173.0463 (M-H-Fer-H_2O). (c) Spectrum for identification of feruloyltyramine. MS/MS experiment was performed in the negative mode, using *m/z* 314.14 as a parent ion. Fragments used for identification: *m/z* 177.0568 (M-H-Tyr), 145.0283 (M-H-Tyr-$HOCH_3$).

Chapter 6

General Discussion and Synthesis

Arjen van Doorn



General Discussion and Synthesis

Co-evolution has shaped many specific interactions between plants and their insect herbivores and over the past 400m years, plants have evolved to specifically respond to chemical cues present in the insect's oral secretions, while specialized insect herbivores have evolved mechanisms to detoxify a wide array of as poisonous plant secondary metabolites. Coevolutionary theory predicts that the huge diversity of plant secondary metabolites has been shaped by herbivory (Ehrlich and Raven, 1964). However, especially the field of plant-pathogen interactions has shown that plants respond very clearly to attack by pathogens by inducing both general and specific defense mechanisms (Chester, 1933; Jones and Dangl, 2006). The ability to induce defenses has been established for algae (Gilbert, 1966), flowering plants (Green and Ryan, 1972) and trees (Schultz and Baldwin, 1982). Induced defenses play an important role in the defense against insect herbivores: Not only do insects perform better when they are allowed to feed on plants that are deficient in induced defenses (Paschold *et al.*, 2007), but herbivorous insects can extend their host range non-host to plants deficient in induced defenses (Kessler *et al.*, 2004; Paschold *et al.*, 2007). This suggests that, for the plant species in which these mechanisms have been shown, induced defenses are critical for their resistance against a wide range of insects.

As previously described, plant-insect interactions are very specific; 90% of all herbivores feed on less then three different plant families (Futuyma and Gould, 1979), something that will also affect the initiation of induced defense responses. From the plant's perspective, the wounding caused by the insect's mandibles, and the subsequent recognition of its OS will be one of the first events that induces the activation of induced defenses. After this recognition, among other responses a signaling cascade will be activated that leads to the rapid accumulation of the plant hormone jasmonic acid (JA). This accumulation is accompanied by a plethora of modifications to this molecule. The JA-Ile conjugate, and perhaps also other modified forms, then bind to a receptor-based complex which triggers transcriptional activation. These jasmonate activated genes will then produce a metabolic rearrangement, with the accumulation of toxic components in the leaves and the alteration of primary metabolism. When the plant is challenged by herbivores in its natural environment, these changes will result in being optimally defended.

In this thesis, I studied several stages important in plant-herbivore interactions: OS-perception, JA accumulation, transcriptional activation and metabolic re-arrangements which together have ecological consequences for plants grown in a natural environment.

FAC metabolism on the wounded leaf surface

In contrast to interactions between plants and pathogens, very little is known about the perception or metabolism of herbivore-derived chemical cues. In Chapter 2, I described the metabolism of the insect elicitor 18:3-Glu on wounded *N. attenuata* leaves, and showed that this elicitor was metabolized in a variety of different 18:3-Glu derivatives. After application of synthetic 18:3-Glu or *M. sexta* OS to wounded leaves, the amounts of recovered 18:3-Glu decreased rapidly: Within 30 sec ~60% was metabolized, indicating that a very rapid metabolism took place on the leaf surface. To discover the fate of this 18:3-Glu, and to test the hypothesis that 18:3-Glu can be cleaved into its fatty-acid and amino-acid moieties by the plant, radioactive ^{14}C-18:3-Glu (labeled in the fatty-acid chain) was applied to wounded leaves and extracts analyzed by TLC and radio-HPLC. These experiments falsified the hypothesis: No free 18:3 could be detected. However, the radio-HPLC analysis showed that 18:3-Glu was metabolized into a large variety of more polar derivatives. Using LC-MS/MS, the oxygenated molecules 13-oxo-13:2-Glu, 13-OH-18:3-Glu and 13-OOH-18:3-Glu were identified. Steam-treating the leaves showed that this process was heat-labile, and corroborating with the identification of the 13-OOH-derivative it was demonstrated that this conversion is mediated by the lipoxygenase NaLOX2; inverted-repeat (ir)*lox2* plants (silenced for NaLOX2) metabolize 18:3-Glu more slowly, and do not produce oxygenated derivatives when 18:3-Glu is applied to wounded leaves. However, when 18:3-Glu was applied to intact leaves, no conversion could be observed, showing that enzymes present in the leaf are responsible for the conversion of 18:3-Glu. This conversion occurs quickly, suggesting that upon leaf rupture the enzyme is present in abundant amounts. NaLOX2's native function is in the production of 13-hydroperoxides that originate from 18:2 or 18:3 fatty acids and which are subsequently cleaved by a hydroperoxide lyase to form C_6 volatiles and C_{12} products (Halitschke *et al.*, 2004). As soon as a leaf (of many, if not all plant species) is damaged, the odor of these C_6 volatiles (aptly named "green leaf volatiles") can immediately be detected, indicating immediate action of NaLOX2 (Allmann *et al.*, 2010). Together, these results strongly suggest that NaLOX2 is responsible for the conversion of 18:3-Glu into its oxygenated forms.

A previous study showed, using beet armyworm caterpillars fed with radiolabeled maize plant material, that the FAC volicitin (17-OH-18:3-Gln) was transferred from the feeding caterpillar to the plant material, but no additional radiolabeled fractions could be recovered from the leaf tissue (Truitt and Paré, 2004). These result suggest that volicitin can not be metabolized by wounded maize leaves, which may be explained by the presence of a 17-OH group on the fatty-acid moiety of volictin; this might inhibit LOX-mediated conversion of this FAC.

Hydroxyperoxide fatty acids are relatively unstable substrates, and the formation of 13-OH-18:3-Glu could be mediated by either an enzymatic, or by a spontaneous reaction. It is plausible that 13-OH-18:3-Glu is derived from 13-OOH-18:3-Glu, either via an enzymatic or non-enzymatic conversion. The formation of 13-oxo-13:2-Glu requires the α-cleavage of the C_{13}-C_{14} bond; in *Glycine max* seeds enzymatic activity which cleaves 13-OOH-18:3 into 13-oxo-trideca-9,11-tridecanoic acid and two isomeric pentenols has been described (Salch *et al.*, 1995).

Application of purified 13-oxo-13:2-Glu and 13-OH-18:3-Glu to wounded leaves showed that 13-oxo-13:2-Glu induced JA 2-fold over wounding, to the same level as 18:3-Glu, showing that this molecule is active in terms of JA biosynthesis activation. To test whether the conversion of 18:3-Glu into 13-oxo-13:2-Glu resulted in a different response, volatiles of ir*lox*2 plants were compared after 18:3-Glu and 13-oxo-13:2-Glu elicitation, and it was shown that β-pinene and an unknown monoterpene were differentially induced by 13-oxo-13:2-Glu, which may indicate that differential activation of monoterpenes is depending on 18:3-Glu metabolism. 13-OH-18:3-Glu however, was not able to induce JA more than wounding alone, indicating that metabolism of 18:3-Glu may also be important for generating inactive forms, possibly to regulate the 18:3-Glu mediated stimulus.

Together, these results suggest that there is an additional layer of complexity in plant-insect interactions, taking place between the larval mandibles and the wounded plant tissue.

Advances in the elucidation of insect elicitor signaling

Although progress has been made in the identification of insect elicitors, compared to the plant-pathogen field, many questions remain unanswered. The exact role insect elicitors play in herbivory has also been questioned and remains controversial. In Chapter 3, we discussed advances in elucidating the role of insect elicitors in plant defense signaling. In plant-pathogen interactions, several pathogen-associated molecular patterns are known, and their mechanisms have been elucidated in great detail (Jones and Dangl, 2006). For insect elicitors, it has been shown that plants respond to elicitors by accumulating the phytohormones JA, salicylic acid and ethylene, and that there is a high degree of specificity in the recognition of insect elicitors (Schmelz *et al.*, 2009). However, insects deposit only nL saliva on wounded leaves (Peiffer and Felton, 2009), and from an evolutionary point of view, the recognition of damaged cells is a more logical choice because it does not allow insects to "escape" recognition by not producing elicitors (Heil, 2009). On the other hand, plants recognize very small amounts of OS: even at a 1:1000 dilution plants respond significantly to a few µL over wounding alone (Schittko *et al.*, 2000). Moreover, it was shown that *N. attenuata* responds differentially to the OS of two different Lepidopteran herbivores, indicating that there is specificity in the response to different herbivores (Diezel *et al.*, 2009). Only relying on "self-

recognition" mechanisms, as proposed by Heil (2009), would remove a layer of information for the plant. Some compounds derived from the insect OS have been labeled as mediators of "damaged self-recognition", and although this can be argued for fragments derived from plant proteins [e.g. inceptins, (Schmelz *et al.*, 2006b)], elicitors based on insect enzymes or on compounds synthesized by insects should not be classified as such. Moreover, the first elicitors discovered in plant-pathogen interactions were also plant-derived compounds, but at present we know that the most important elicitors in the interaction between plants and pathogens are pathogen-derived compounds.

As described before, the FACs 18:2-Glu and 18:3-Glu are the major elicitors in *M. sexta* OS, and *N. attenuata* responds to the perception of these elicitors with a significant metabolic rearrangement [see (Gaquerel *et al.*, 2009; Giri *et al.*, 2006; Halitschke *et al.*, 2003)], and recently the first steps were made in elucidating the signaling pathways elucidated by 18:3-Glu. *N. attenuata* responds with differential JA accumulation when 18:3-Glu is applied to wounded tissue, but the mechanism by which this occurs is unclear. In a recent study, Kallenbach *et al.* (2010) looked at what step in the JA biosynthesis pathway 18:3-Glu acts, and concluded that it is likely that regulation happens very early in the pathway, at the step of 13-hydroperoxidation. In another study, transcriptional elements specifically induced by 18:3-Glu were studied by a non-targeted transcriptional analysis, which came up with a large number of 'tags' that were specifically upregulated after 18:3-Glu was applied to wounded leaves (Gilardoni *et al.*, 2010).

Combined with the study presented in Chapter 2, we conclude that although insect elicitors have been identified from a relatively wide variety of herbivores, we are still very much scratching the surface when it comes to uncovering the mechanisms regulating the initiation of the defense response in plants. Untargeted approaches to identify early regulated genes and metabolites may lead to the elucidation of induced signal transduction pathways, and subsequently silencing key genes in these pathways and analyzing the response after treatment with insect elicitors will bring the field substantially forward. Forward-genetics strategies using *A. thaliana* can also be employed to elucidate signaling mechanisms in this plant.

Regulation of jasmonic acid metabolism in S. nigrum

Wounding and deposition of OS on the wounded leaf tissue often elicits the accumulation of JA and conjugates, and this accumulation is pivotal to the activation of many defense mechanisms. However, by what mechanisms JA is metabolized, or how the JA accumulation is regulated is less well studied. We know that there is great heterogeneity in the jasmonate response among even closely related plant species. I decided to dissect jasmonate signaling in the weedy Solanaceous species *Solanum nigrum*; previously it was show that this plant's transcriptome responded differently than

N. attenuata to attack by the same Solanaceous specialist herbivore, *M. sexta* (Schmidt *et al.*, 2005), and I was interested to investigate differences in *M. sexta*-induced jasmonate signaling in these plants.

To investigate this, three genes in the JA signaling cascade were targeted for RNAi-mediated gene-silencing: SnLOX3 to target JA biosynthesis, SnJAR4 to target JA conjugation to JA-Ile and SnCOI1 to target the jasmonate recognition machinery. Jasmonate profiling showed that after OS elicitation, JA accumulated in a double burst; wounding elicited an initial burst of 400 ng g^{-1}, and OS elicitation resulted in a second burst at 90 min, at which the JA levels increased 3 to 4-fold over wounding alone. Induction of 12-hydroxy jasmonates was not immediately induced, and after 60-90 minutes these jasmonates were 4 to 5-fold induced by OS. The main burst of JA-Ile however, was elicited after 30 minutes, but did not differ between wounding and OS treatment. This was followed by a second burst at 2h after treatment that was smaller than the first, but induced by OS.

These results are different from *A. thaliana* where JA and JA-Ile peak both at 3h and stay high for at least 24h (Glauser *et al.*, 2008). In *N. attenuata,* OS induced a JA increase above above wounding-induced levels within 15 minutes and peak at 45 to 60 minutes (Kallenbach *et al.*, 2010), while JA-Ile follows the JA burst (Wang *et al.*, 2007).

As expected, jasmonate accumulation in *S. nigrum* was dependent on SnLOX3: JA, 12-OH-JA and 12-OH-JA-Ile levels were reduced to less than 2% of WT plants. However, ir*lox3* plants had 30% as much JA-Ile as WT plants, indicating that maintenance of JA-Ile signaling is prioritized by the plant. JA-Ile biosynthesis is regulated by SnJAR4: JA-Ile levels were reduced to 15% of WT in these lines.

Although OS-induced changes in JA appeared after 90 minutes in *S. nigrum* WT plants, ir*jar4* plants showed differential JA and JA-Ile levels as early as 30 minutes, showing that OS also influences early jasmonate accumulation. The OS-induced JA burst is induced by SnCOI1: ir*coi1* plants did not accumulate more JA after OS treatment than after wounding.

However, JA-Ile levels are not regulated by SnCOI1; JA-Ile levels accumulated to greater then WT levels in these plants. The turnover of JA-Ile, a previously proposed COI1-dependent mechanism for JA-Ile regulation, was not changed in *S. nigrum* ir*coi1* plants, and the expression of SnJAR4 was also not changed in ir*coi1* plants. This indicated that other mechanisms are responsible for the high JA-Ile levels in these plants.

The 12-hydroxy forms of JA were first discovered as tuber-inducing compounds (Koda and Okazawa, 1988), but 12-OH-JA was recently proposed to negatively regulate the expression of JA biosynthesis genes, thus playing a role as negative regulator of JA biosynthesis (Miersch *et al.*, 2008). Accumulation of 12-OH-JA was similarly reduced in ir*coi1* and ir*jar4 S. nigrum* plants, demonstrating

that both jasmonate perception by SnCOI1 and JA-Ile accumulation are necessary for the regulation of 12-hydroxylation. 12-OH-JA-Ile was also downregulated in ir*coi1* and ir*jar4*. 12-Hydroxylation of JA-Ile is probably regulated by SnCOI1, while in ir*jar4* plants the low levels of 12-OH-JA-Ile are likely a combined effect of downregulated 12-hydroxilation and JA-to-Ile conjugation activity.

JA-glucose was identified using [2,3-^{3}H]JA and [1,2-^{13}C]JA as an additional OS-induced jasmonate in *S. nigrum*. JA-glucose was originally described in tobacco bright-yellow-2 cells after JA feeding (Qian *et al.*, 2004) and was also detected as wound-induced jasmonate in in *A. thaliana* plants (Glauser *et al.*, 2008). Accumulation of JA-glucose in *S. nigrum* was strongly reduced in ir*lox3* and ir*coi1*, but not in ir*jar4* plants, in which wound-induced levels were augmented compared to WT. These results showed that the regulation of JA-glucosidation, in contrast to 12-hydroxilation, is only dependent on SnCOI1, but not on JA-Ile. This suggests that in *S. nigrum*, SnCOI1 can act independently from JA-Ile.

Activation of systemic signaling is depending on the second, OS-induced jasmonate burst

Plants do not only activate defense responses in wounded tissue; defense-related compounds also accumulate in non-wounded systemic tissue after damage is perceived (Green and Ryan, 1972). I investigated the relationship between the local jasmonate kinetics and the activation of systemic signaling. The enzyme leucine amino peptidase (LAP) is a direct defense trait in *S. nigrum* (Hartl *et al.*, 2008). To study the influence of the temporal dynamics of jasmonate accumulation on systemic LAP activation, local leaves were elicited, and leaves ablated at different time points, and systemic LAP levels were analyzed after 48h. The LAP eliciting signal that travels from the local to the systemic leaf needed between 90 and 240 min to leave the local leaf, and required OS elicitation; no induction of LAP activity was observed in systemic leaves after wounding. ir*lox3* and ir*coi1* plants showed no activation of systemic LAP after OS elicitation, demonstrating the importance of jasmonate production and perception in the activation of systemic signaling. Systemic LAP levels in ir*jar4* plants, were not different from WT plants, and because ir*lox3* plants accumulated twice the JA-Ile levels as ir*jar4* plants, suggest these results that local JA-Ile accumulation is not essential for the activation of systemic signaling. Studies using grafted tomato plants were congruent to ours; local jasmonate accumulation is essential for production of the systemic signal (Li *et al.*, 2002). Studies in *A. thaliana* however, showed that systemic JA and JA-Ile levels increased very rapidly (Glauser *et al.*, 2009; Koo *et al.*, 2009), while in *S. nigrum* basal levels in systemic leaves were ca 20 ng g^{-1} FW increasing to 80 ng g^{-1} FW after OS elicitation, which is 40 times less than in local tissue. Systemic JA-Ile accumulation was minor (2 to 3 ng g^{-1} FW), and was not differentially induced by W+OS treatment. Because JA accumulation in the systemic leaf was only activated after 180 min, the

movement or activation of systemic JA is slower in *S. nigrum* than in *A. thaliana*, even when the greater traveling distance in *S. nigrum* is considered.

We conclude that that jasmonate metabolism is dependent SnCOI1, but only partly on SnJAR4 and JA-Ile, while the activation of systemic signaling depends on jasmonate production and perception, but not on JA-Ile.

Transcriptomic, metabolomic and ecological effects of jasmonate signaling.

After the perception of herbivory and the subsequent jasmonate response, a large transcriptional response is elicited. The plant activates genes that code for a plethora of defense and tolerance mechanisms, resulting in a metabolic re-arrangement and thus, in a plant that is better defended against insect herbivores. In Chapter 4, I showed that the local accumulation of JA-Ile did not play an essential role in the activation of a systemic defense, and in Chapter 5, I asked what role jasmonate production, conjugation of JA to JA-Ile and perception played in the activation of rapid gene expression, the metabolic re-arrangement, and what consequences this has for the plant in a natural environment. Transcriptional profiling was analyzed using the TIGR potato microarray; previous studies showed that the TIGR potato microarray could be used for hybridizations with *S. nigrum* cDNA (Schmidt *et al.*, 2005). An early time point was chosen to analyze jasmonate-activated genes: W+OS-elicited leaves from WT, ir*lox3,* ir*jar4* and ir*coi1* plants were harvested after 3h, mRNA was extracted and cDNA was synthesized. In individual experiments, cDNA of WT was hybridized against ir*lox3*, ir*jar4* or ir*coi1*. The results showed that in ir*jar4* plants, six genes were regulated compared to WT plants. Five of these genes were related to drought or salt stress in other plant species. Specifically, the homologue of AtHB12 in *N. attenuata*, NaHD20, was shown to be downregulated in ir*lox3*, ir*jar4* and ir*coi1* plants. Transcriptional profiling of *N. attenuata* plants moreover, showed that ir*jar4/6* plants had reduced expression of defense-related genes such as proteinase inhibitors (PIs) and polyphenol oxidases. PIs were also represented on the TIGR chip used in our study, but not regulated in ir*jar4* plants compared to WT. ir*lox3* and ir*coi1* plants regulated a large number of genes, ca 700 genes were regulated in both genotypes. In both genotypes, most defense-related genes were downregulated, while most photosynthesis-related genes were upregulated compared to WT. This was similar to other plant species where was shown that JA biosynthesis and perception was essential for the induction of defense-related genes after wounding (Halitschke and Baldwin, 2003). The increase in transcription of photosynthesis-related genes indicated that the herbivore-induced reduction in photosynthesis after herbivory (Zangerl *et al.*, 2002) is JA-dependent, as reported before (Zavala and Baldwin, 2006).

Subsequently, we performed a metabolic analysis on *S. nigrum* plants, and surprisingly, no glycoalkaloids could not be detected in the leaves of the plant, unlike studies that have detected these in *S. nigrum* plants. However, in green berries these compounds were present in high amounts, indicating that there is high variability of glycoalkaloid accumulation among different *S. nigrum* genotypes. To test *S. nigrum*'s metabolic response after after OS elicitation, WT, ir*jar4* and ir*coi1* plants were elicited by W+OS and leaves harvested after 12, 24 and 48h, together with an untreated control at t = 0. Leaf extracts were analyzed by high-resolution LC-TOF and peaks were extracted using XCMS (for a detailed description, see the Materials and Methods section in Chapter 5). However, many ions belong to a single compound, and a correlation-based method to cluster these together was developed, which reduced the number of 2889 ions to 1430 clusters. Interestingly, of all clusters regulated in WT, only 6% of these genes were also regulated in both ir*jar4* and ir*coi1*, showing that jasmonate signaling plays a predominant role in the activation of the metabolic rearrangement after OS elicitation. Moreover, analyzing the data at individual time points revealed that ir*jar4* and ir*coi1* have at 48h a 50% overlapping profile, but not at 12 and 24h. This indicates that at early time points SNJAR4 and SNCOI1 signal independently, while after 48h the interaction between SNJAR4 and SNCOI1 cause a large portion of the changes.

To identify ions that might play a role in jasmonate-mediate herbivore defense, we selected ions which were regulated over time, between any of the used genotypes, and changed 1.5-fold between any genotypes, at any time point. This resulted in the identification of 60 ions of interest, which were searched in databases, and in some cases elucidated in MS/MS experiments. Based on these MS/MS and database searches we could identify JA-12-O-Glc and two members of the phenylpropranoid pathway; feruloyltyramine and feruloylquinic acid. The latter two compounds have been described as wound-induced in tomato, but jasmonate independent (Pearce *et al.*, 1998). The initial accumulation of both compounds was dependent on SNJAR4 and SNCOI1, but after 48h no difference between WT, ir*jar4* and ir*coi1* levels could be observed, suggesting that jasmonate signaling is important for the initial accumulation, but that the late induction is jasmonate-independent. The identification of JA-12-O-Glc showed that jasmonate accumulation is not only activated for the few hours described in Chapter 4, but continues for at least two days following OS elicitation. The accumulation pattern is similar to that described for 12-OH-JA: ir*coi1* plants accumulate very little, while ir*jar4* plants have ~50% reduced levels of JA-12-O-Glc (see Chapter 4).

The relevance of these metabolic changes was investigated by transplanting ir*lox3*, ir*jar4*, ir*coi1* and WT plants into a field plot in the Southwestern United States. Within nine days of transplanting the plants into the field, ir*lox3* and ir*coi1* plants were heavily attacked by herbivores:

They experienced 4 to 5-fold more *Noctuidea* damage than WT plants, while ir*jar4* suffered from a similar amount of damage as WT plants. These differences could not be linked to oviposition differences: When the initial damage was scored, it was the same between all lines, indicating that the differences in herbivore damage were due to differences in the defense response of the plant. Mortality in the field was also significantly higher in ir*lox3* and ir*coi1* plants; after eight days of transplantation only 78 and 22% of the plants survived, respectively. ir*jar4* plants on the other hand, survived as well as WT plants: 49 plants out of the 50 planted survived. In has been reported before that plants silenced for *LOX3* and *COI1* have reduced defenses, resulting in increased herbivory levels in the field (Kessler *et al.*, 2004; Paschold *et al.*, 2007), but plants with reduced levels of JA-Ile have not been tested in a natural environment before. However, Wang *et al.* (2008) showed that *M. sexta* performs better on *N. attenuata* ir*jar4/6* plants, while PI and nicotine levels were reduced in these plants. PI levels in field-grown *S. nigrum* were not different, which indicated that JA-Ile signaling in *S. nigrum* plays a different role than the initiation of direct defenses.

The results mean that the large metabolic reorganization displayed by the ir*jar4* plants do not have a strong effect on defense responses in a field condition. However, the transcriptional profiling showed that 5 of the 6 regulated genes were involved in drought or salt stress, which presents the hypothesis that SnJAR4 plays a role in regulating drought or salt stress responses in *S. nigrum.*

Induced defenses in a co-evolutionary context

The early experimental work of Ernst Stahl (1888), and the development of co-evolutionary theory by Erhrlich and Raven (1964) have lead to a model that proposes that plant secondary metabolites have shaped herbivore speciation events. However, a number of studies with plants mutated or silenced in components of the jasmonate signaling cascade have shown that not only the species-specific secondary metabolites, but also the ability to induce defense is essential for plant survival. These jasmonate-deficient plants are not deficient in their constitutive defense: when *M. sexta* caterpillars were moved regularly to systemic leaves of *N. attenuata* ir*coi1* and WT plants, they performed the same on both genotypes (Paschold *et al.*, 2007). However, when ir*coi1* plants were transplanted into the field, not only performed existing herbivores better on the plants, new herbivores could also be observed feeding on them (Paschold *et al.*, 2007), as was reported for plants silenced in Na*lox3* (Kessler *et al.*, 2004).

In the plant species studied so far, it is clear that induced defenses play a very significant role in plant defense, and because plants of a wide taxonomic variety respond to MeJA we can assume that jasmonate signaling is a ubiquitous signaling pathway for the activation of induced defenses. In this thesis, the discovery of insect elicitor modification indicates that plants can regulate induced

defenses already in a very early phase of attack, while the wide variety of jasmonate metabolites, and their highly variable accumulation and metabolism patterns indicate that specific induced defenses are regulated by jasmonate metabolism. Also, this provides further evidence for the hypothesis that the specificity in plant-herbivore interactions is very high, a specificity that is evident after the first bite.

The importance of induced defenses under natural conditions has been described in a limited number of species and an even more limited number of plant genera, but the existence of these induced defenses has been described for a wide variety of plant species. Thus, if we want to understand the role of plant secondary metabolites in the co-evolution between plants and herbivorous insects, we should especially consider the metabolites and defense traits induced by the feeding of these insects.

Zusammenfassung

Nach 400 Millionen Jahren Koevolution haben Pflanzen und Insekten verschiedene Verteidigungs- und Gegenverteidigungsmechanismen entwickelt. Seit etwa 100 Jahren sind pflanzliche Sekundärmetabolite als wichtige Komponenten in der Pflanzenverteidigung bekannt. Soweit die Pflanze jedoch weitgehend mit diesen giftigen Stoffen gefüllt ist, kann sich diese effektive Verteidigung auch negativ auf nützliche Insekten auswirken. Des Weiteren ist die konstitutive Synthese solcher Metabolite sehr energieintensiv, insbesondere wenn die Pflanze nicht von Insekten befallen wird.

Auf diese Problemstellungen reagieren die meisten Pflanzen mit einer induzierten Verteidigung. Wenn Verteidigungsmechanismen nur bei Insektenbefall aktiviert werden, ist dies kosteneffizienter und minimiert die negativen Einflüsse auf Nützlinge. Damit diese induzierten Verteidigungsmechanismen funktionieren, muss das fressende Insekt erkannt und ein Signalübertragungsweg aktiviert werden, welcher letztendlich die Gentranskription aktiviert. Diese Gene dekodieren Mechanismen, die eine Gegenreaktion auf den Fressfeindbefall darstellen. Die geänderte Transkription dieser Gene führt zu Veränderungen im Primär- und Sekundärmetabolismus der Pflanze, wodurch die Pflanze dann meist besser verteidigt ist. In dieser Dissertation habe ich mich mit der Erkennung von chemischen Signalen im Oralsekret (OS) von Insekten und dem Metabolismus von Jasmonsäure beschäftigt. Insbesondere habe ich untersucht wie sowohl die Jasmonsäurebiosynthese als auch die Jasmonsäureerkennung die Gentranskription und das Metabolom von Pflanzen beeinflussen, um letztendlich die Wirkung des Metabolismus von Jasmonsäure auf den Fressfeindbefall im Feld festzustellen.

Ich konnte zeigen, dass das Fettsäure-Aminosäure-Konjugat 18:3-Glu, welches den hauptsächlichen Signalstoff im OS der Manduca sexta darstellt, schnell an der Blattwunde metabolisiert wird. Höchstwahrscheinlich produziert eine von der Lipoxygenase LOX2 abhängige, enzymatische Reaktion das Intermediat 13-OOH-18:3-Glu, welches danach in 13-OH-18:3-Glu und 13-oxo-13:2-Glu umgewandelt wird. In Versuchen, in denen ich Pflanzen mit den beiden letztgenannten aufgereinigten Stoffen behandelt habe, konnte ich feststellen, dass 13-oxo-13:2-Glu und 18:3-Glu jeweils dieselbe Menge an Jasmonsäure im Blatt induzierten, da 13-OH-18:3-Glu inaktiv war. Zudem wurde auch erkannt, dass die Monoterpenbiosynthese von 13-oxo-13:2-Glu höher angeregt wird als von 18:3-Glu. Dies ist ein Hinweis darauf, dass der Metabolismus von Insektenelizitoren auf der verwundeten Blattoberfläche eine Rolle in der Feinregelung der Verteidigungsreaktion der Pflanze spielt. Diese Ergebnisse deuten auf eine zusätzliche

Komplexitätsebene in der Pflanze-Insekt-Interaktion hin, die zwischen der Mandibula der Raupe und dem verwundeten Gewebe besteht.

Jasmonate sind entscheidende Signalmoleküle in der Interaktion zwischen Pflanze und Insekt, jedoch unterscheiden sich die charakteristischen Anreicherungsmuster von Jasmonaten bereits zwischen nahe verwandten Pflanzenarten. Aus diesem Grund habe ich mich entschieden, die Signalkaskade von Jasmonaten in einer noch nicht charakterisierten Pflanzenart, *Solanum nigrum*, zu untersuchen. Mithilfe transgener Pflanzen, in denen die Gentranskription von SnLOX3, SnJAR4 und SnCOI1 minimiert ist, habe ich den Jasmonsäuremetabolismus in Pflanzen analysiert, die Jasmonsäure entweder nicht synthetisieren, nicht zu Isoleucin konjugieren oder nicht erkennen können. Als Ergebnis stellte sich heraus, dass der Jasmonsäuremetabolismus von SnCOI1 und SnJAR4 abhängig ist, da in beiden Genotypen weniger 12-OH-Jasmonate akkumulierten. Weitere Experimente ergaben die Abhängigkeit der 12-Hydroxylierung von SnCOI1 und JA-Ile. Jasmonsäure-Glukose wurde mithilfe radioaktiver und stabiler Isotope identifiziert, wobei die Akkumulation dieses Jasmonats nur von SnCOI1 und nicht von JA-Ile abhängig war. Die Rolle der lokalen Jasmonateakkumulation wurde in Bezug auf die Aktivierung systemischer Verteidigungsmechanismen untersucht. Ich konnte aufzeigen, dass die systemische Verteidigung nur aktiviert wird, soweit das lokale Blatt mit OS behandelt wurde und das verantwortliche Signal daraufhin das lokale Blatt innerhalb der Zeitspanne von 90 bis 240 Minuten nach der Verwundung verließ. Eigene Studien mit transgenen Pflanzen ergaben, dass die Jasmonsäureproduktion und die Jasmonsäureerkennung für die Aktivierung der systemischen Verteidigung essentiell sind und Jasmonsäure-Isoleucin nur eine untergeordnete Rolle spielt.

Durch die Wahrnehmung von Insektenbefall und die Aktivierung des Jasmonsäure-Signalübertragungsweges werden viele Gene transkriptionell aktiviert. Um diese transkriptionellen Änderungen zu untersuchen, wurden Microarrays mit Blattproben drei Stunden nach Verwundung und OS-Applikation hybridisiert. Diese Versuche ergaben, dass SnLOX3 und SnCOI1 ca. 700 Gene regulierten und SnJAR4 dagegen lediglich sechs Gene steuerte. Dieses Ergebnis verdeutlicht, dass die durch Insekten induzierten, transkriptionellen Änderungen von Jasmonsäurebiosynthese und -erkennung jedoch nicht von der Konjugation der JA zu JA-Ile beeinflusst werden.

Insektenbefall führt auch zu einer Veränderung des Metaboloms der Pflanze, wodurch die Pflanze besser vor Fressfeinden geschützt ist. Von besonderem Interesse war für mich der Einfluss von Jasmonaten auf diese metabolomischen Änderungen. Aus diesem Grund habe ich das Metabolom von ir*jar4*- und ir*coi1*-Pflanzen nach Verwundung und OS-Applikation zu unterschiedlichen Zeiten verglichen. Diese Analyse zeigte, dass SnJAR4 und SnCOI1 zusammen für 94% der induzierten Ionen

nach Verwundung und OS-Behandlung verantwortlich waren. Vergleicht man die Ionenprofile von SNJAR4- und SNCO11-Pflanzen, zeigt sich eine Überschneidung der Profile von ungefähr 50%, was darauf hinweist, dass ein Teil der Induzierung von SNCO11 bzw. SNJAR4 unabhängig ist. Zusammengenommen bestimmen all diese Faktoren wie gut die Pflanze in ihrer natürlichen Umgebung vor Insektenfraß geschützt ist. In Feldexperimenten mit diesen transgenen Pflanzen im Südwesten Amerikas konnte ich feststellen, dass SNLOX3 und SNCO11 nicht nur für den Schutz gegen Fressfeindbefall, sondern auch für das Überleben in der natürlichen Umgebung essentiell sind.

Die Basis der Evolutionären Theorie, die von den Pionieren der chemischen Ökologie begründet worden ist, hat die Bedeutung der zahlreichen pflanzlichen Sekundärmetabolite für die Evolution von Pflanze-Insekt-Interaktionen und die Artenentstehung bereits herausgestellt. Neuere Entwicklungen der letzten 100 Jahre haben gezeigt, dass die Pflanze hierbei allerdings keine passive Rolle einnimmt, sondern aktiv auf Insektenfraß reagiert. Studien mit transgenen Pflanzen, welche nur bedingt auf Insektenbefall reagieren können, haben gezeigt, dass auch Insekten, die normalerweise nicht auf dieser Pflanzenart fressen können, in der Lage sind diese Pflanzen zu schädigen. Hieraus wird deutlich, dass die induzierte Verteidigung eine tragende Rolle in der Verteidigung der Pflanze einnimmt. In dieser Dissertation habe ich beschrieben, wie wichtig die Metabolisierung von Insektenelizitoren und Jasmonsäure für diesen Prozess ist. Wenn wir verstehen möchten wie pflanzliche Verteidigungsmechanismen die Interaktion zwischen Pflanze und Insekt beeinflussen, ist es von entscheidender Bedeutung, die für die induzierte Verteidigung verantwortlichen Vorgänge zu untersuchen.

Summary

After 400 million years of co-evolution, plants and insects have developed a wide variety of defense and counter-defense strategies. Plant secondary metabolites have been known for over 100 years to be very important in plant defense, but filling the plant with toxic metabolites, although effective in defense, has detrimental effects on beneficial insects, and is metabolically costly in the absence of herbivores.

Induced defenses address these problems; activating defense traits only upon insect attack is efficient and minimizes "friendly fire". However, for this induced defense to function, the feeding insect needs to be efficiently recognized, after which a signaling cascade needs to be activated which subsequently activates gene transcription. These genes encode for the mechanisms beneath the herbivore response and the changed gene transcription will lead to changes in primary and secondary metabolism, which eventually produces a better-defended plant. In this thesis, I asked questions about the recognition of chemical cues in the insect's oral secretions, the metabolism of jasmonic acid (JA), and finally how jasmonic acid biosynthesis, metabolism and perception influence large-scale gene transcription, and finally how the metabolism of JA influences herbivory in the field.

Here, I showed that the main elicitor in *M. sexta*'s oral secretions (OS), the fatty-acid amino acid conjugate (FAC) 18:3-Glu, is rapidly metabolized on wounded leaves. An enzymatic reaction involving the lipoxygenase LOX2 most likely produced the intermediate 13-OOH-18:3-Glu, which was subsequently metabolized into 13-OH-18:3-Glu and 13-oxo-13:2-Glu. In elicitation experiments, it was shown that 13-oxo-13:2-Glu induced JA to the same levels as 18:3-Glu, while it differentially induced monoterpene biosynthesis, indicating that this conversion fine-tunes the defense response of the plant.

Jasmonic acid is a crucial signal molecule in plant-insect interactions, but its accumulation patterns and metabolism is vary greatly between even closely related plant species. Therefore, we decided to dissect jasmonate signaling in a previously uncharacterized plant species, *Solanum nigrum*. Using transgenic plants silenced for SnLOX3, SnJAR4 and SnCOI1, I analyzed JA metabolism in plants without JA biosynthesis, JA conjugation and JA perception. My results showed that SnCOI1 and SnJAR4 played an important role in the metabolism of JA, both genotypes accumulated less 12-OH-jasmonates, which was shown to be dependent on SnCOI1 and JA-Ile signaling. JA-glucose was identified using radio- and stable isotopes, and the accumulation of this jasmonate was dependent on SnCOI1, but not JA-Ile, indicating that there are COI1-dependent, JA-Ile jasmonate signaling mechanisms in *S. nigrum*. We could show that systemic defenses were only activated when insect oral secretions were applied to wounds on the local leaf, and that the signal for systemic signaling leaves

the local leaf between 90 and 240 minutes after elicitation with W+OS. Studies with the transgenic plants showed that JA production and perception were essential in the activation of systemic defenses, while JA-Ile played only a minor role.

To assess the large transcriptional activation that occurs after the perception of herbivory and the subsequent activation of the jasmonate signaling cascade, we conducted a microarray 3h after OS treatment, these showed that in ir*coi1* and ir*lox3* a large proportion transcripts accumulated differentially compared to WT, while ir*jar4* plants only showed minor differences compared to WT. In a metabolomic analysis, we compared ir*jar4* and ir*coi1* to WT plants at different time points after OS elicitation, and could show that SnJAR4 and SnCOI1 were together responsible for 94% of the ions that were induced over time. However, the metabolic profiles of ir*jar4* and ir*coi1* only partly overlapped, and 50% of the SnJAR4 regulated ions were SnCOI1-independent, showing that there is both COI1-dependent, JA-Ile-independent signaling, as well as JA-Ile-dependent, COI1-indepedent signaling. To test how these changes would affect the plant in a natural environment, we planted ir*lox3*, ir*jar4* and ir*coi1* plants along with WT in the field, and estimated herbivory levels. We saw that SnLOX3 and SnCOI1 play a very important role in defense against *Noctuidea* herbivores, while SnJAR4 were not more damaged than WT plants.

Coevolutionary theory proposes that the great abundance of plant secondary metabolites has shaped, and has been shaped by plant-insect interactions, and has shaped speciation events. However, it has been shown that induced defenses are essential in plant defense, and that insects normally not able to feed on a plant may do so when the plant is unable to induce defense, indicating that induced defenses are also important in shaping plant-insect interactions. In this thesis, I described that plant metabolism of insect elicitors in ways that can tune the induced defense response, and that jasmonic acid is metabolized by different mechanisms into several jasmonates, which may be important in the evolution of induced defenses. If we want to understand how defense traits in plants have shaped plant-insect interactions we should take a careful look at induced defenses, and the pathways that regulate them.

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Declaration of Independence

Ich erkläre, entsprechend § 5 Abs. 3 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena, dass mir die geltende Promotionsordnung der Fakultät bekannt ist. Ich habe die Dissertation selbstständig und nur unter Zuhilfenahme der im Text angegebenen Quellen und Hilfsmittel angefertigt, wobei alle von Dritten übernommenen Textabschnitte entsprechend gekennzeichnet wurden. Personen, die zu den Experimenten, der Datenauswertung oder der Verfassung der einzelnen Manuskripte beigetragen haben, sind in die Manuscript overviews unter Angabe ihrer jeweiligen Beiträge zur Arbeit aufgeführt, oder werden, im Falle von Beiträgen geringeren Ausmaßes, in den Danksagungen am Ende der entsprechenden Manuskripte genannt. Gemäß Anlage 5 zum § 8 Abs. 3 wurde die Beschreibung des von mir geleisteten Eigenanteils von Prof. Ian T. Baldwin, dem Betreuer der Dissertation, mit Unterschrift bestätigt und der Fakultät bei Einreichung dieser Dissertation vorgelegt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde von mir niemals zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Desweiteren habe ich keine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

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VanDoorn, A, Baldwin, IT & Bonaventure G (2010) Lipoxygenase-mediated modification of insect elicitors: Generating chemical diversity on the leaf wound surface
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Accepted for publication in *New Phytologist*

VanDoorn A, Bonaventure G, Rogachev I, Aharoni, A, Schmidt, DD & Baldwin, IT
JA-Ile signaling in *Solanum nigrum* is not requited for defense responses in nature.
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